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BIOLOGICAL OXIDOREDUCTIONS[1,2]

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Problems related to cellular oxidoreductions continue to occupy a central and highly active field in biochemical research, with the levels of experimental approach ranging from the isolated enzyme to the intact organism. The enzymological aspects of the topic have been dealt with extensively in the preceding volume of this series (1), and a treatise on oxidoreduction enzymes has just appeared in two recent volumes of *The Enzymes* (2). Oxygenases, which were the subject of this chapter two years ago (3), are further reviewed in a special volume (4). A survey of the chemistry and function of coenzyme Q has also been published recently (5). In addition metalloproteins are treated in a separate chapter (7) of this volume.

In view of these facts it was considered appropriate to devote the present review to oxidoreduction reactions and related processes as they occur at a more integrated level of biological organization, namely, in the mitochondrion. Particular attention will be focused on the energy-linked reversal of electron transport in the respiratory chain as well as on the reaction pathways and intermediates of mitochondrial energy transfer. Significant progress in these fields has been made since they were last reviewed in this series (8), and a new summing up at this stage therefore appears justified.

ORGANIZATION OF THE RESPIRATORY CHAIN

The well-recognized association of cellular respiration with the mitochondrial membrane system, and the repeated observation of constant molar relationships among individual electron transport catalysts in mitochondria from various tissues and species (9–12), have stimulated a great deal of interest in the structural and functional organization of the respiratory chain. Two types of experimental approach have principally been used: kinetic studies of the respiratory chain *in situ* in mitochondria and submitochondrial particles; and reconstruction of the electron transport system from isolated enzymes.

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[1] Review of the literature was completed in December 1963.

[2] The following abbreviations are used throughout this chapter: AcAc (acetoacetate); As_i (arsenate); ADP (adenosine diphosphate); ATP (adenosine triphosphate); BAL (2,3-dimercaptopropanol); CoQ [coenzyme Q (ubiquinone)]; CTP (cytidine triphosphate); DNP (2,4-dinitrophenol); DT diaphorase (dicoumarol-sensitive flavoenzyme); EDTA (ethylenediametetraacetate); ETP (electron transport particle); GTP (guanosine triphosphate); ITP (inosine triphosphate); NAD^+, NADH (nicotinamide adenine dinucleotide, oxidized and reduced form); $NADP^+$, NADPH (nicotinamide adenine dinucleotide phosphate, oxidized and reduced form); P_i (inorganic orthophosphate); TMPD (NNN′N′-tetramethyl-*p*-phenylenediamine); UTP (uridine triphosphate). Other abbreviations are explained in the text.

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On the basis of kinetic studies, Chance & Williams (9) have concluded that the catalysts of the respiratory chain, although limited in their movement by the structure, function as discrete components, interacting by way of simple thermal collision. More recently, the term "oxysome" has been introduced (13) to represent the concept of a macromolecular assembly of enzymes capable of carrying out all the functions of electron transport and related processes. Although the suffix "-some" usually refers to a morphological entity, a "body," the oxysome is meant to denote a functional concept, according to which the catalysts of the respiratory chain are distributed within or over the surface of the mitochondrial membrane system such that each possesses some individual mobility. Thus, for example, a given molecule of cytochrome-*b* could interact with any number of cytochrome-c_1 molecules of equal proximity.

A somewhat different mechanism has been proposed by Green and associates (12, 14–26) on the basis of reconstruction studies. According to these authors, the respiratory chain consists of four "fixed" components, viz., NADH-CoQ reductase ("Complex I"), succinate-CoQ reductase ("Complex II"), $CoQH_2$-cytochrome-*c* reductase ("Complex III"), and cytochrome oxidase ("Complex IV"); as well as two "mobile" components, viz., CoQ and cytochrome-*c*. The fixed components are not single catalysts but are complexes, Complex I consisting of NADH dehydrogenase and nonheme iron; Complex II of succinate dehydrogenase, nonheme iron, and cytochrome-*b*; Complex III of cytochrome-*b*, nonheme iron, and cytochrome-c_1; and Complex IV of cytochrome-*a* and copper. Each of these has been isolated and purified (20–24), and the molar ratios of their content of individual catalysts have been established (12). It was further shown (11, 14–19) that the four complexes could be recombined in a 1:1:1:1 molar ratio to form a unit which exhibited integrated electron transport activity, provided that the two mobile components, CoQ and cytochrome-*c*, were present. Recombination required a high concentration of at least one of the four complexes; but, once it had taken place, dilution had no effect. Lipids were also essential for the recombination (25, 26). This picture of the organization of the respiratory chain differs from that of Chance and associates (9, 10, 13) in that only at two sites, CoQ and cytochrome-*c*, can the chain operate by random collision, the remaining sites of interaction being fixed within the individual complexes. Thus, a molecule of CoQ may interact with any molecule of cytochrome-*b*, but a molecule of cytochrome-*b* may interact with only one given molecule of cytochrome-c_1, the one present within the same complex.

A further restriction in this pattern of mobility comes from the recent introduction by Green and associates (14, 15, 16, 27, 28) of the concept of the "elementary particle." This concept arose from two developments: (*a*) the discovery by Fernândez-Morán (29, 30) of a repeating structural unit in the mitochondrial membrane; and (*b*) the isolation by Criddle et al. (31) of a protein, called "structural protein," which is devoid of oxidation-reduction groups and which accounts for more than 50 percent of the total protein of beef heart mitochondria or electron transport particles derived therefrom. Prompted by these observations, Blair et al. (32) isolated from beef heart mitochondria, treated with a combination of bile salts and ammonium sulfate, a particulate fraction which catalyzed the aerobic oxidation of NADH and succinate at rates 2–2.5 times higher than did sonicated mitochondria or electron transport particles. The concentration of the "fixed" electron transport components was increased by a factor of about 2.2. The particles, which appeared homogeneous in the ultracentrifuge, had an estimated molecular weight of 2.1×10^6. Further purification by removal of residual structural protein led to an estimated minimum molecular weight of 1.4×10^6, but also decreased the enzymic activity. The value of 1.4×10^6 was identical with that calculated for the reconstructed four-complex system (12). Hence it was concluded (32) that the isolated particles were a physical and functional aggregate of the four complexes constituting the respiratory chain. Furthermore, it was postulated (14, 15, 16, 27, 28) that both the isolated and the reconstituted particles were identical with the repeating unit observed by Fernândez-Morân (29, 30) in intact mitochondria, and the name "elementary particle" was proposed for this entity.

The introduction of this concept means a further restriction in the degree of freedom of the respiratory chain carriers, implying a fixed arrangement not only within the individual complexes but also with regard to complex-complex interactions. However, the postulated relationship between the repeating units and the respiratory chain is not yet supported by published experimental evidence, and a consideration of its functional implications would therefore appear premature. The morphological observations of Fernândez-Morân (29, 30) have been confirmed and extended in several laboratories (33–37), even though the interpretations as to the size, shape, and spacing of the repeating units seem to vary from one investigator to another. Also, similar units have been observed in membranes of nonmitochrondrial origin (14, 34, 35) as well as in mitochondria from animal species deficient in various cytochromes (38).

In 1958, Keilin & King (39, 40) reported the reconstitution of succinoxidase by means of soluble succinate dehydrogenase and alkali-treated Keilin-Hartree heart-muscle preparation. King (41) later showed that the reconstitution took place regardless of whether the succinate dehydrogenase and the alkali-treated heart-muscle preparation were derived from the same or different species.

These conclusions have been criticized by Singer and associates (42, 43, 44), mainly on the ground that the alkali-treated preparation contained the same amount of succinate dehydrogenase flavin as did the preparation before the alkali treatment. They postulated that the restoration of succinoxidase activity observed by Keilin & King (39, 40) was caused by a reactivation of this succinate dehydrogenase rather than a reconstitution by means of the added enzyme. However, King (39, 40, 45, 46) could demonstrate that there was a precise stoichiometry between reincorporated added succinate dehydrogenase and resulting succinoxidase activity, and that this relationship held also when the adhering, inactive, succinate dehydrogenase was partially removed from the alkali-treated preparation by recentrifugation prior to neutralization.

Whatever may be the exact mechanism of the restoration, both groups of workers now seem to agree that only succinate dehydrogenase prepared under special precautions can exhibit this effect (42, 43, 45–48), and that the restorative ability of the isolated enzyme is more labile towards treatments such as aging than is any of its known catalytic parameters (44, 46, 49).

More recently, King et al. extended their studies by isolating what they called a cytochrome-*b*-c_1 complex (50) and a cytochrome-*c* cytochrome oxidase complex (51). The cytochrome-*b*-c_1 complex contained cytochrome-*b*, cytochrome-c_1, nonheme iron, and coenzyme Q, in the approximate molar proportions of 1:0.6:2.6:0.3, as well as 19 percent lipid. The cytochrome-*c* cytochrome oxidase complex contained cytochrome-*c* and cytochrome oxidase in a 1:1 molar ratio. When the cytochrome-*b*-c_1 complex was combined with soluble succinate dehydrogenase, an active succinate cytochrome-*c* reductase system was obtained (52). Combination of the cytochrome-*b*-c_1 and cytochrome-*c* cytochrome oxidase complexes with soluble succinate dehydrogenase resulted in an active succinoxidase system (53). These results of King et al. are rather similar to those of the Madison group, even though they indicate that the distinction between "fixed complexes" and "mobile components," as visualized by the Madison group, may be somewhat arbitrary and dependent on the experimental tactics used for the separation and recombination of the individual constituents. In the system of King et al., all components, including coenzyme Q and cytochrome-*c*, appear to be parts of fixed complexes. However, the work of both groups strongly supports the concept of an extensive ability for complex formation among individual respiratory chain enzymes, suggesting that electron transport may not in all instances require intermolecular collisions but may proceed by an interaction of electron-carrying prosthetic groups, made possible physically by reversible conformational changes of a single apoprotein complex. The much-debated question of cytochrome-$a+a_3$ (cf. 54, 55) may be a case in point, where kinetic evidence suggests the existence of two hemes but all efforts to separate two apoenzymes chemically so far have failed.

In an excellent review of the 40-year-old history of the respiratory chain, Nicholls (56) concludes:

> Until the individual components of the cytochrome chains are isolated in enzymically active form, it will be difficult for treatises such as the present one to consider them in separate chapters. In fact, such a method of review may contribute to a false picture of the nature of cytochromes. For, however extensive our knowl-

edge may become, any discussion of these enzymes must consider the fact that they react with one another and not with other metabolites. It is not possible to describe cytochrome *b* without considering flavo-proteins and cytochrome c_1; nor is it reasonable to discuss cytochrome oxidase without mentioning the properties of cytochrome *c*. A classification under the name—"the enzymes"—is limiting our ideas.

The history of the study of cytochromes, the history of the study of respiration, a study traditionally influential in determining our ideas about the nature of life, contains many examples of such limiting names and concepts. The model systems, such as the charcoal and palladium models, distorted ideas in so far as they enshrined such concepts. At least the relation between concept and model, as in the computer program, is today explicitly recognized. The enduring problem underlying the discussions and theories about cytochrome organization is the question whether a living system can be analyzed exhaustively by a consideration of the chemistry of its components. This, the dispute of Pasteur and Liebig, remains unsettled. The reactions of the cytochromes within the mitochondrion provide a fertile field for expounding both points of view.

RELATIONSHIP OF ELECTRON AND ENERGY TRANSFER AS REVEALED BY STUDIES WITH INHIBITORS AND UNCOUPLERS

Agents which inhibit electron or energy transfer, or uncouple the two processes, continue to serve as powerful tools in the study of mitochondrial respiration and oxidative phosphorylation. Superficially, these agents may be divided into three categories: inhibitors of electron transfer, inhibitors of energy transfer, and uncoupling agents. However, there is considerable overlapping among these effects, and, in fact, several of the inhibitors recently studied focus attention on this very aspect. In addition, a number of new and powerful agents have been introduced.

In a study of the mode of action of antimycin A on rat-liver mitochondria and on nonphosphorylating submitochondrial particles from pigeon heart, Estabrook (57) has confirmed the earlier conclusion of Chance (58) that antimycin A blocks electron transport between cytochromes-*b* and -*c*. With the pigeon heart particles, a molar ratio of approximately 1 was found between antimycin A titer and cytochrome-*b* content, which is in accordance with the value earlier established by Chance (59) for the Keilin-Hartree heart-muscle preparation. With the liver mitochondria, on the other hand, the molar amount of antimycin A required for complete blockage of the respiratory chain was about one third of the content of cytochrome-*b*. From analog computer analysis of the observed kinetics of the reduction of cytochrome-*b* and of the inhibition of O_2 consumption during the titration with antimycin A, Estabrook (57) also concluded that the carrier with which antimycin A interacts probably is not cytochrome-*b*, but is the oxidized carrier subsequent to cytochrome-*b*, possibly nonheme iron. This possibility had earlier been considered by Tappel (60). Estabrook (57) further found that the antimycin A titer of the mitochondria was equal under phosphorylating and nonphosphorylating (uncoupled) conditions, indicating that the effect of antimycin A on electron transport is independent of the functional state of the energy transfer system. On the other hand, antimycin A has been reported to exhibit effects on energy transfer, such as a stimulation of the latent mitochondrial ATPase (61, 62), and an inhibition of the P_i-ATP exchange (63, 64).

Howland (65) reported that the inhibition of succinate oxidation in rat-liver mitochondria by 2-heptyl-4-hydroxyquinoline-N-oxide and 2-hydroxy-3(3-methyl-2-butyl)-1,4-naphthoquinone (hydrolapachol), both of which block electron transport between cytochrome-*b* and cytochrome-*c*, is partially reversed by low concentrations of DNP. It was concluded that these compounds act on both energy-conserving and electron transport reactions. In accordance with the work of Estabrook (57), no effect of DNP was found on the antimycin A inhibition, but it was pointed out that this may be due to the extremely tight binding of antimycin A to its target compound in the mitochondria. In a further study, Howland (66) found that increasing concentrations of both 2-heptyl-4-hydroxyquinoline-N-oxide and antimycin A lowered the P/O ratio with ascorbate+TMPD (i.e., reduced cytochrome-*c*) as the electron donor in rat liver mitochondria. Without the inhibitors, the P/O ratio was slightly but significantly above 1, in accordance with earlier observations of Ramirez & Mujica (67, 68).

These findings were interpreted to indicate that the aerobic oxidation of reduced cytochrome-*c* might be connected with more than one phosphorylation. Recently, however, Lee et al. (69) demonstrated that TMPD is able to establish a bypass of the antimycin A-sensitive site of the respiratory chain, transferring electrons from reduced cytochrome-*b* to cytochrome-*c*. Although this shunt circumvents one phosphorylating site of the respiratory chain, an increase of the P/O ratio with ascorbate plus TMPD might arise from simultaneous oxidation of NAD^+-linked endogenous substrates via TMPD.

Conover et al. (70, 71, 72) have carried out a series of investigations into the functional relationship of the dicoumarol-sensitive flavoenzyme, called DT diaphorase, to the respiratory chain in rat liver mitochondria and submitochondrial particles. This enzyme, which has earlier been purified and studied in detail by Ernster et al. (73), catalyzes the oxidation of both NADH and NADPH by various benzo- and naphthoquinones and redox dyes but not by cytochrome-*c*. On the basis of these and a series of other features, it has been concluded (73, 74, 75) that the DT diaphorase in all likelihood is identical with the vitamin-K reductase described earlier by Martius (76–79), who has postulated that this enzyme is a component of the phosphorylating respiratory chain. Although DT diaphorase occurs mainly in the soluble fraction of the rat liver homogenate, a small portion of the enzyme is associated with the mitochondria. Conover et al. (70, 71, 72) have found that this mitochondrial DT diaphorase can establish a bypass over the amobarbital-sensitive site of the respiratory chain, provided that a catalytic amount of vitamin K_3 is added. The DT diaphorase-vitamin K_3 mediated respiration of the amobarbital-inhibited mitochondria was highly dicoumarol sensitive, and it proceeded via cytochrome-*b* as revealed by spectrophotometric evidence and by inhibition with antimycin A. The P/O ratio was one unit lower than in the amobarbital-free system. Interestingly, only vitamin K_3, out of a number of quinones tested, was efficient in exhibiting this effect, despite the fact that all of them were active electron acceptors for soluble DT diaphorase. This could not be the result of inaccessibility of the mitochondrial DT diaphorase to these quinones, since, when external cytochrome-*c* was added, most of the quinones were active in mediating the DT diaphorase-linked bypass of the amobarbital-sensitive site, and the respiration now was also insensitive to antimycin A. Furthermore, the same observations were made when mitochondria or DT diaphorase-free submitochondrial particles were combined with purified DT diaphorase, using added NADH or NADPH as the hydrogen donor (71, 72). From the collected evidence (70–75) it was concluded that DT diaphorase or vitamin-K reductase in all probability is not a component of the phosphorylating and respiratory system, but that vitamin K itself may be involved in the mitochondrial NADH oxidase in accordance with the original suggestion of Martius & Nitz-Litzow (80).

Ernster et al. (81, 82) have investigated the effect of the fish-poison, rotenone, on rat liver mitochondria. In confirmation of earlier observations of Fukami & Tomizawa (83, 84) and Lindahl & Öberg (85, 86), they found that rotenone is a powerful inhibitor of mitochondrial respiration supported by pyridine nucleotide-linked substrates, but does not inhibit the respiration associated with succinate. This pattern of selective inhibition is similar to that found with amobarbital (87). Unlike amobarbital, but like antimycin A, rotenone was found to be firmly bound to the mitochondria (or submitochondrial particles), and its effect on respiration was not relieved by dilution or centrifugation of the suspensions. The molar amount of rotenone required for complete inhibition of respiration was approximately 25 mμmoles per gram of liver-mitochondrial protein, or about one half of the corresponding amount of antimycin A. The rotenone titer appears to be the lowest ever reported for an electron transport inhibitor, and it also appears to be lower than the concentration of any known respiratory chain catalyst, suggesting that a hitherto unknown catalyst of the order of 25 mμmoles per gram of protein may be involved between NAD^+ and cytochrome-*b* in the liver-mitochondrial respiratory chain. In addition, titration with rotenone revealed a nearly linear relationship between the amount of rotenone added and the extent of inhibition of the oxygen uptake (in contrast to the markedly sigmoid titration curve for antimycin A), which indicates that the rotenone-sensitive catalyst probably is the rate-limiting component of the mitochondrial NADH oxidase. Ernster et al. (81, 82) also compared the effects of rotenone and amobarbital on energy transfer reactions. Rotenone did not share the earlier-reported effects of amobarbital on the DNP-induced ATPase (62, 88, 89) and the P_i-ATP exchange reactions (64), effects that had been interpreted (64, 88, 89) as evidence for the involvement of an NAD^+-flavin electron shuttle in these reactions. It was concluded (81, 82) that rotenone inhibits electron transport only, whereas amobarbital inhibits both electron transport and energy transfer.

Pumphrey & Redfearn (90, 91) have reported that high concentrations of amobarbital partially inhibit the aerobic oxidation of succinate in both tightly coupled and DNP-uncoupled mitochondria from rat liver. Disruption of the mitochondria by treatment with Ca^{++} abolished the inhibition. Amobarbital also lowered the P/O ratio, in accordance with earlier observations (92, 93, 94).

In an interesting series of experiments, Chance and associates (95, 96) investigated the effects of amobarbital and of some other known inhibitors of NAD^+-linked respiration with respect to their effect on energy transfer. They found that the concentration of amobarbital required for half-inhibition of NAD^+-linked respiration was about three times higher with uncoupled than with phosphorylating mitochondria. In the latter case, this concentration was considerably lower than those earlier reported by Ernster et al. (87) (whose system involved the operation of the entire Krebs cycle, including the oxidation of succinate), but was rather close to the value reported by Aldridge & Parker (97, 98). Chance & Hollunger (96) also found that the amobarbital-induced reduction of the mitochondrial pyridine nucleotide was much less extensive in uncoupled than in phosphorylating rat liver mitochondria. In pigeon heart mitochondria the extent of pyridine nucleotide reduction was the same under phosphorylating and uncoupled conditions, and the only difference found was that the amobarbital concentration required to reach maximal reduction was higher in the uncoupled than in the coupled system. This probably reflects the difference in $NADP^+$ content between the two types of mitochondria. Among the other compounds studied, progesterone and methylene glycol were shown to act in a fashion similar to that of amobarbital, whereas thiopental exhibited both an amobarbital-like action and a pronounced uncoupling effect.

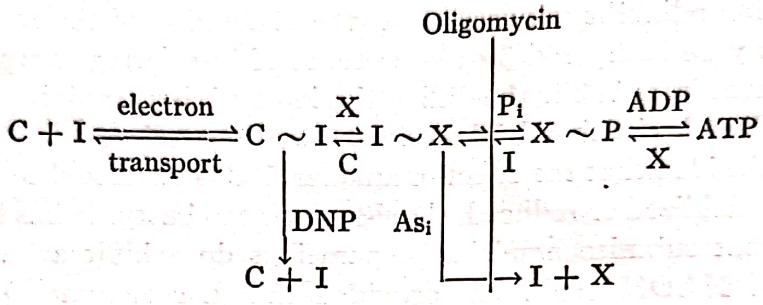
It was concluded that this class of agents combines an inhibition of electron transfer between flavin and cytochrome-*b* with an inhibition of energy transfer at the NADH and flavin site. The inhibition of energy transfer causes increased pyridine nucleotide reduction and leads to increased flavoprotein oxidation in phosphorylating preparations. This hypothesis provides a reasonable explanation for the previous findings that amobarbital acts between NAD^+ and flavin in phosphorylating mitochondria (99) and between flavin and cytochrome-*b* in nonphosphorylating submitochondrial particles (100).

In their studies of a soluble NADH dehydrogenase from beef heart electron transport particles, Minakami et al. (101) have found that the oxidation of NADH by this enzyme, as measured with ferricyanide as the electron acceptor, is insensitive to amobarbital. From this finding, and from the observation that NADH oxidation by ferricyanide also is amobarbital-insensitive in the parent electron transport particles, they postulated that the NADH dehydrogenase reaction cannot be the site of amobarbital inhibition in intact mitochondria. It was pointed out that the spectrophotometric evidence on which the conclusion regarding the mitochondrial localization of the site of amobarbital action between NAD^+ and flavin was based (99) may be of questionable reliability, inasmuch as the evaluation of the redox state of the flavin may be hampered by the presence of interfering compounds, primarily nonheme iron. While the difficulties in obtaining a reliable estimate of the redox state of mitochondrial flavin are well recognized (cf. 102), the acceptance of the idea that the effect of amobarbital is unrelated to NADH dehydrogenase, oxidized or reduced, should not be too hasty. It has been shown repeatedly that submitochondrial preparations do exhibit an amobarbital sensitivity of NADH oxidation, provided that a coenzyme Q derivative (103–105) [or, in general, a 2-methyl-quinone (71, 74)], rather than ferricyanide, is used as the electron acceptor. Even if these substances are usually less active acceptors than ferricyanide, they may be more closely related chemically to the natural neighbor of NADH dehydrogenase in the respiratory chain.

Moreover, Estabrook (106) has reported that both amobarbital and antimycin A inhibit ferricyanide reduction by NAD^+-linked substrates in intact mitochondria. This interesting finding points to the possibility that reactivity with ferricyanide may not be a property of the native NADH dehydrogenase but may emerge upon disruption of the mitochondrial structure; the existence of a permeability barrier as a reason for the nonreactivity appears to be improbable in view of the high rate of ferricyanide reduction observed in the absence of amobarbital and antimycin A. The ready tendency of NADH dehydrogenase to undergo modifications is known from recent experience (107–114).

Oligomycin, introduced in 1958 by Lardy et al. (115), is presently one of the most valuable tools in the study of mitochondrial energy transfer. This antibiotic is a powerful inhibitor of tightly coupled mitochondrial respiration, but does not interfere with the electron transport of loosely coupled or uncoupled mitochondria or submitochondrial particles (115, 116). In loosely coupled mitochondria, oligomycin abolishes phosphorylation (116), and the available evidence is consistent with the conclusion that oligomycin inhibits ATP formation from all three coupling sites of the respiratory chain. It inhibits the mitochondrial P_i-ATP (115) and $O^{18}P_i$-H_2O (117) exchange reactions, as well as the mitochondrial ATPase reactions as induced by various agents (117), including arsenate (118).

On the other hand, arsenate, unlike DNP and related uncouplers, does not relieve the inhibitory effect of oligomycin on tightly coupled respiration (116, 119). This is an important feature since it permits a differentiation of the site of action of DNP and related uncouplers from that of arsenate. Furthermore, it shows that oligomycin in all likelihood acts prior to the intervention of P_i in the sequence of energy transfer steps leading to ATP formation. These relationships may be pictured by the following reaction scheme where C is an electron carrier (oxidized or reduced), and I and X are energy transfer carriers:



Bruni et al. (120, 121) and Vignais et al. (122) have studied the effects of atractyloside (or potassium atractylate, a glucoside from *Atractylus gummifera*) on the oxidative phosphorylation and related reactions of mitochondria and submitochondrial particles. This compound appears to share most of the effects of oligomycin, with the interesting exception that these are only observed above a certain level of structural organization of the mitochondria. Vignais et al. (122) visualized that a bypass of a reaction step in the energy transfer sequence of the disrupted mitochondria may be responsible for the lack of atractyloside sensitivity. These findings are of great significance because they may open an insight into the mechanism of those functional modifications, such as loss of respiratory control or emergence of ATPase activity, which are well-known consequences of the disruption of the mitochondrial structure.

Another striking parameter of this type is the mitochondrial ADP-ATP exchange reaction studied by Wadkins & Lehninger (123–125). This reaction is sensitive to DNP and oligomycin only in tightly coupled mitochondria, not in submitochondrial particles or with the soluble ADP-ATP exchange enzyme. Addition of the latter to digitonin particles restores the effects of the inhibitors, whereas addition of low concentrations of azide to mitochondria or digitonin particles abolishes the DNP and oligomycin sensitivities of the ADP-ATP exchange reaction. On the basis of these findings, Wadkins & Lehninger (125) proposed an extension of the hypothesis of Vignais et al. (122) and suggested that the observed functional modifications upon disruption of the mitochondrial structure may arise from the dissociation of an enzyme complex, M·E, into M and E. Thus, in the tightly coupled mitochondria, the energy transfer would proceed according to the sequence:

$$\mathrm{C} \sim \mathrm{M{\cdot}E} \overset{\mathrm{P_i}}{\rightleftharpoons} \mathrm{P} \sim \mathrm{M{\cdot}E} \rightleftharpoons \mathrm{M{\cdot}E} \sim \mathrm{P} \overset{\mathrm{ADP}}{\rightleftharpoons} \mathrm{ATP} + \mathrm{M{\cdot}E}$$

(C = electron carrier), whereas in the loosely coupled submitochondrial particles the reaction sequence would be:

$$C \sim M \overset{P_i}{\rightleftharpoons} P \sim M \overset{E}{\rightleftharpoons} P \sim E \overset{ADP}{\rightleftharpoons} ATP + E$$

In the tightly coupled mitochondria, the ADP-ATP exchange would involve the reaction:

$$ATP + M \cdot E \rightleftharpoons M \cdot E \sim P + ADP$$

and, in the loosely coupled submitochondrial particles, the reaction:

$$ATP + E \rightleftharpoons ADP + P \sim E$$

If it is assumed that, in both cases, oligomycin blocks the step involving P_i and DNP acts by splitting the intermediate preceding this step, these schemes would account for the observed loss of sensitivity of the ADP-ATP exchange towards oligomycin and DNP upon disruption of the mitochondria. They do not account, in their present form, for the differential effects of DNP and arsenate on oligomycin-inhibited respiration. Further aspects of this problem will be discussed in the section on oxidative phosphorylation. The phosphorylating and respiratory control properties of submitochondrial particles have been studied by Gregg & Lehninger (126–128) and by Haas & Elliot (129).

In 1955 Hollunger (130) presented a detailed study of the effects of various guanidines on mitochondrial oxidative phosphorylation and related reactions. Like amobarbital, these compounds inhibited NAD^+-linked, but not succinate-linked, respiration. However, in contrast to the amobarbital effect, the respiratory inhibition was readily relieved by DNP. Recently, the mode of action of guanidines has been studied very extensively by Chance & Hollunger (131), Chappell (132), and Pressman (133–135). From spectrophotometric evidence, Chance & Hollunger (131) concluded that guanidine binds a nonphosphorylated high-energy intermediate of the phosphorylation occurring in the NAD^+-flavin region. A similar conclusion was reached by Chappell (132) concerning 4-methyl-3-butanyl guanidine and hexylguanidine. Pressman (133, 135) found that alkyl guanidines inhibit, with increasing potency according to the chain-length of the substituent, the respiration of rat liver mitochondria with glutamate+malate as substrate. DNP, but not dicoumarol or 2,6-dinitro-4-isooctylphenol, relieved the inhibition. This differed from the effect of oligomycin which was relieved by all three uncouplers. Alkyl guanidines also did not inhibit DNP-induced ATPase, in contrast to oligomycin. From the lack of inhibition of the DNP-induced ATPase, Pressman visualized that the guanidines might act on an intermediate located between the respiratory chain and the DNP-sensitive site. Furthermore, he suggested that dicoumarol and dinitro-octylphenol may not share the ability of DNP to uncouple the NAD^+-linked phosphorylation, since these compounds did not release the guanidine inhibited respiration. However, as pointed out by Chappell (136), the splitting of the guanidine-bound high-energy intermediate may not be critical from the point of view of the overall rate of DNP induced ATPase. Likewise, the lack of effect of dicoumarol and dinitrooctylphenol in restoring the guanidine-inhibited respiration may not be incompatible with a general uncoupling effect of these compounds, if one assumes that they do not split the guanidine-bound high-energy intermediate although they do split the same intermediate in the absence of guanidine. Pressman (134) has also reported that guanidine enhances the active uptake of P_i into mitochondria, and is incorporated itself into the mitochondria in a manner competitive with the incorporation of Mg^{++}. Guanidines were also found to inhibit the Ca^{++}-activated ATPase in mitochondria (130).

The long-standing problem of the mode of action of DNP and related uncouplers is not yet settled. The subject has been reviewed extensively by Slater (137). There is general agreement that these agents act prior to the intervention of P_i in respiratory chain-linked energy transfer. The findings with oligomycin, outlined above, also eliminate the nonphosphorylated high energy intermediate immediately preceding the intervention of P_i (denoted above as $I \sim X$) as the site of DNP action, since this intermediate, logically, is the one split by arsenate. Hence, unless there exist more than two nonphosphorylated high-energy intermediates in the reaction sequence, there remains $C \sim I$ as the site of action of DNP.

Hülsmann (138), in 1958, suggested that both uncouplers and certain respiratory inhibitors act by reaction with a high-energy intermediate of this type, according to the reactions:

$$C \sim I + \phi \rightleftharpoons C + \phi - I$$

$$\phi - I \rightleftharpoons \phi + I$$

(where ϕ denotes the uncoupler or inhibitor). He proposed that the difference between an uncoupler and a respiratory inhibitor (such as amobarbital or antimycin A) is a quantitative one, depending upon the relative ease of the decomposition of ϕ–I by the second reaction. This hypothesis has received strong support from recent studies of the effects of amobarbital and antimycin A, reviewed earlier in this section, as well as from studies of Chance et al. (139) who investigated the spectrophotometric and respiratory effects of uncoupling agents. They found that low concentrations of uncouplers induced changes in the steady state of the respiratory carriers that were more rapid than those elicited by P_i and ADP, suggesting that the uncouplers react with an intermediate closer to the respiratory chain than the intermediate with which P_i and ADP react (i.e., that they react with $C \sim I$ rather than $I \sim X$). High uncoupler concentrations caused an inhibition of respiration, and this effect was interpreted in terms of an association between the uncoupler and I in accordance with the hypothesis of Hülsmann (138).

If $C \sim I$ were the site of action of uncouplers, there should exist three uncoupler-induced ATPases, one for each coupling site of the respiratory chain. Furthermore, since $C \sim I$ contains an electron transport carrier in either the oxidized or the reduced form, the activities of these ATPases would be influenced by the redox state of the respiratory chain. Despite much experimental work (cf. 137), the evidence available is not clear regarding these points. On the basis of studies with amobarbital and with flavin inhibitors Löw et al. (62, 64, 88, 89) have proposed that the DNP-induced ATPase involves predominantly one coupling site of the respiratory chain, the one in the NAD$^+$-flavin region. However, rotenone, which inhibits electron transport in the same region as amobarbital, does not share the effects of amobarbital on the DNP-induced ATPase (82). Moreover, guanidines do not inhibit the DNP-induced ATPase (130, 133), although they inhibit energy transfer related to the NAD$^+$-flavin-linked phosphorylation (131–134). On the basis of pH-activity curves, Myers & Slater (61, 140) postulated the existence of three DNP-induced ATPases, but these findings were later reinterpreted by Hemker (141), who concluded that it is not possible to identify more than one DNP-induced ATPase with this method. More recently Hemker (142) demonstrated two ATPase activity maxima in rat liver mitochondria with increasing concentrations of DNP, one at 0.1 mM and another at 0.25 mM DNP. Amobarbital suppressed the maximum at 0.1 mM DNP, and antimycin A suppressed the one at 0.25 mM DNP. The two effects were largely additive, and 0.8 mM amobarbital+10 μg/mg protein of antimycin A together inhibited the ATPase activity to an average extent of 73 percent. From these results Hemker (142) concluded that the first and second coupling sites of the respiratory chain contribute to the bulk of the DNP-induced ATPase, with a quantitative preponderance of the first site. These conclusions, again, do not account for the observations with rotenone and guanidines, quoted above. In addition, the amount of antimycin A required for obtaining the 73 percent inhibition in the presence of amobarbital was 100 to 200 times greater than that sufficient for maximal inhibition of the respiration. A conceivable, but not yet explored, explanation of the apparent lack of striking responses of the DNP-induced ATPase to changes in the redox state of the respiratory chain is that, when the carriers are in the redox state unfavorable for the formation of C$\sim$I, the concentration of available I increases, and this condition triggers an uncoupler-induced dissociation of I$\sim$X by the reactions

$$I \sim X + \phi \rightleftharpoons X + \phi - I$$

$$\phi - I \rightleftharpoons \phi + I$$

Dissociation of I$\sim$X has been considered to be involved in the mechanism of the so-called Mg^{++}-activated ATPase reaction of damaged mitochondria (cf. 137). Löw (88, 89) has found that low concentrations of atebrin and chlorpromazine inhibit this reaction and simultaneously increase the sensitivity of the DNP-induced ATPase towards amobarbital in a dramatic fashion.

Although it is widely assumed that uncouplers act by splitting a preexisting high-energy intermediate, the alternative possibility, that they may act by preventing high-energy bond formation, has also been considered.

Such a mechanism has been proposed (64, 143, 144) for the energy coupling reaction occurring in the NAD+-flavoprotein span of the respiratory chain. It is based on the consideration that the oxidation of NADH by flavoprotein (Fp) involves, formally, the splitting of a molecule of water:

$$\mathrm{NADH + Fp + HOH \rightleftharpoons NAD^+ + FpH_2 + OH^-}$$

It was visualized (cf. 144, 145) that, in the lipophilic environment in which this reaction takes place in the mitochondrial membrane, a compound of the type IOH would "substitute" for water, the reaction thus taking the form:

$$\mathrm{NADH + Fp + IOH \rightleftharpoons NAD^+ + FpH \sim I + OH^-}$$

(Alternatively, IOH may be split into $\mathrm{IO^-}$ and $\mathrm{H^+}$, with the formation of NAD$\sim$OI; hence, the hypothesis does not intrinsically implicate Fp as part of the high-energy intermediate.) It was further visualized that, when a liposoluble acid (ϕOH)—such as DNP—is present, this might replace IOH, thus allowing electron transport to proceed without the formation of a high-energy intermediate:

$$\mathrm{NADH + Fp + \phi OH \rightleftharpoons NAD^+ + FpH_2 + \phi O^-}$$

(alternatively, again, NAD-Oϕ may be formed, with the subsequent dissociation into $\mathrm{NAD^+}$ and $\phi\mathrm{O^-}$). Data of Hemker (141) have lent some support to this hypothesis, by showing that liposolubility and the undissociated state of nitrophenols are critical for the uncoupling power. Strong evidence in favor of the prevention type of mechanism, however, comes from recent work of Pinchot (146), who has found that DNP prevents the formation of an "$\mathrm{NAD^+}$-enzyme" complex in respiring bacterial particles; but, once the complex had been formed, DNP had no effect on its further reaction with $\mathrm{P_i}$ and ADP to yield ATP; evidence was presented that DNP acted by preventing the association of the "enzyme" with the respiratory particle. Similar findings were reported by Webster et al. (147) concerning a "cytochrome-*c*-factor" complex formed in a phosphorylating beef heart cytochrome oxidase system.

An interesting aspect of the uncoupling effect of pentachlorophenol has been revealed by recent studies of Weinbach et al. (148). They found that this uncoupler is firmly bound to the mitochondria but can be removed therefrom by a stoichiometric amount of serum albumin. The removal resulted in a restoration of oxidative phosphorylation. Serum albumin also promoted a reversal of the swelling caused by pentachlorophenol, but this effect also required ATP. The amount of serum albumin needed for promoting contraction of the mitochondria was much smaller than that needed for restoration of oxidative phosphorylation. It was concluded that pentachlorophenol is bound to various proteins of the mitochondria and that serum albumin competes with these proteins in binding pentachlorophenol. Partial removal of the uncoupler is sufficient to liberate the protein essential for contraction, possibly a contractile protein, whereas all the uncoupler must be removed in order to liberate the sites for energy coupling. A protective effect of serum albumin against the uncoupling action of DNP has been noticed earlier (94, 149).

Heytler and co-workers (150–152) have described a new class of potent uncoupling agents consisting of halogen-substituted carbonyl cyanide phenylhydrazone. The *m*-chloro derivative has been investigated in detail with respect to its effects on respiration, phosphorylation, exchange reactions, ATPase, and relation to the site of action of oligomycin. In all these respects, the effects were similar to those of DNP except that the concentrations required were two to three orders of magnitude lower. Interestingly, the effects of the carbonyl cyanide phenylhydrazones were found to be efficiently counteracted by cysteine and by other 1,2-, as well as 1,3-, aminothiol compounds; but glutathione was inactive.

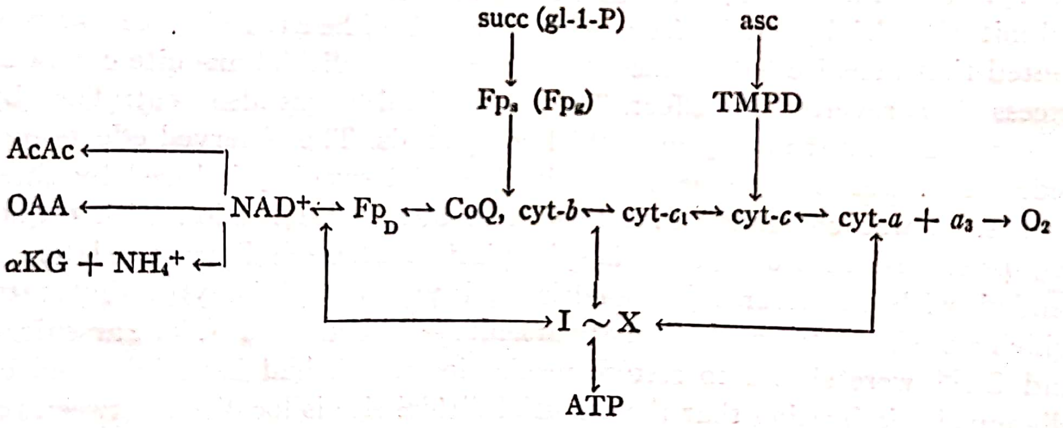
Another powerful group of uncouplers is the phlorobutyrophenone derivative, desaspidin, and related anthelminthic agents, which have been studied in great detail by Runeberg (153). These compounds are firmly bound to the mitochondria and exhibit a markedly diphasic effect on the resting respiration and ATPase, stimulating at low, and inhibiting at high, concentrations.

Fletcher et al. (154–155) demonstrated that arsenite in the presence of 2,3-dimercaptopropanol (BAL), as well as Cd^{++}, uncouples oxidative phosphorylation, inhibits P_i-ATP exchange, and stimulates latent ATPase in submitochondrial particles from rat liver and beef heart. Evidence was presented that arsenite+BAL exert this effect as the dithiol arsenite complex; excess BAL reversed the effect. The effect of Cd^{++} was also neutralized by excess of BAL but not by monothiol compounds. The observed effects preceded, and were independent of, mitochondrial swelling induced by these agents. It was concluded that they were due to interaction with dithiol groups essential for oxidative phosphorylation. These findings were later extended with rat liver mitochondria and γ(*p*-arsenylphenyl)-*m*-butyrate, which was shown to act similarly to arsenite+BAL and Cd^{++}. The arsenicals and Cd^{++} were shown to restore respiration which had been inhibited by oligomycin, indicating that the essential dithiol site is localized between the respiratory chain and the oligomycin-sensitive energy transfer reaction.

In a series of very instructive experiments, Itada & Cohn (156) have followed the fate of O^{18}-labeled arsenate ($O^{18}As_i$) during the arsenolytic ATPase reaction catalyzed by a purified 3-phosphoglycerate kinase—glyceraldehyde-3-phosphate dehydrogenase system and by rat liver mitochondria. With the first system, which represents a substrate-level phosphorylation, one oxygen of the P_i formed was found to be derived from the $O^{18}As_i$, as anticipated from the known mechanisms of the kinase and dehydrogenase reactions. In the mitochondrial system, less than 2 percent of the oxygen of P_i was derived from $O^{18}As_i$, indicating that the reaction mechanism of the phosphorylation sequence differs from that in the model system. On the other hand, the mitochondria catalyzed a rapid exchange between $O^{18}As_i$ and H_2O, the rate of which was 50 to 100 times greater than the overall rate of ATP hydrolysis in the same system. The relative rates followed the same pattern as the relative rates of $O^{18}P_i$-H_2O exchange with respect to the rates of both oxidative phosphorylation and P_i^{32}-ATP exchange. Some implications of these results will be considered in the section on oxidative phosphorylation.

REVERSAL OF ELECTRON TRANSPORT IN THE RESPIRATORY CHAIN

It is now firmly established that mitochondria and submitochondrial particles from a variety of tissues catalyze an energy-dependent reversal of electron transport through the respiratory chain. The energy for the reaction can be supplied either by added ATP or by endogenous high energy intermediates generated by the respiratory chain. In the former case, the reaction involves an actual reversal of oxidative phosphorylation, and there is now evidence that electron transport through all carriers of the respiratory chain, from NAD^+ to cytochrome oxidase, can be reversed in this way. When endogenous high-energy intermediates generated by the respiratory chain serve as the source of energy, the reversal of electron transport can proceed directly at the expense of these intermediates, without the involvement of the phosphorylating system. The two types of energy-dependent reversal of electron transport and their relation to oxidative phosphorylation are illustrated in Scheme 1.



SCHEME 1. Energy-dependent reversal of electron transport in the respiratory chain, and its relation to oxidative phosphorylation.

Fp_D = NADH dehydrogenase; Fp_s = succinate dehydrogenase; Fp_G = glycerol-1-phosphate dehydrogenase; succ = succinate; gl-1-P = glycerol-1-phosphate; asc = ascorbate; cyt = cytochrome; AcAc = acetoacetate; OAA = oxaloacetate; αKG = α-ketoglutarate.

SYSTEMS STUDIED

Both types of energy-dependent reversal of electron transport in the respiratory chain have been studied under a great variety of conditions, summarized in the following.

In systems of the first type, mitochondria (157–170) or submitochondrial particles (171–185) were incubated, usually under nonrespiring conditions (in the presence of cyanide or sulfide, or anaerobically), whereupon the carriers of the respiratory chain were reduced. As electron donors for the reduction, either endogenous substrates (157–162, 171, 185) or, when such were not present (as in certain types of mitochondria or in sonic submitochondrial particles), an added substrate was used; in most cases succinate has been the added substrate (157, 158, 162–181), but other electron donors, such as glycerol-1-phosphate (162), ascorbate+CoQ_1 (182, 183), ascorbate+vitamin K_3 (182, 183), ascorbate+TMPD (177, 184), or reduced cytochrome-*c* (171, 185), have also been employed. In order to observe a reversed electron flow upon the addition of ATP, it is further necessary to provide an electron acceptor on the substrate end of the respiratory chain. In sonic particles, which are devoid of $NAD,^+$ this has been done by added NAD^+ (171–184); in mitochondria, it has been done by adding an NADH-oxidizing substrate such as acetoacetate (159–162, 166), oxaloacetate (159–162), or α-ketoglutarate +NH_4 (159–162, 167–169); in certain types of mitochondria and in digitonin particles, NAD^+ is not, or is only partially, reduced in the absence of ATP, and thus no added electron acceptor is necessary. Reversed electron flow upon the addition of ATP can be observed by measuring the changes in redox state of cytochromes-*a*, -*c*, and -*b*, and of coenzyme Q, flavin, and NAD^+. In addition, when NADH-oxidizing metabolites were added, the disappearance of these, or the appearance of their reduced products, has also been determined. The observed changes were followed as functions of time or of the "phosphate potential," i.e., the $[ATP]/[ADP][P_i]$ ratio (or both). In experiments with intact mitochondria, ATP-dependent reversal of electron transport has also been studied under respiring (in some instances under partially azide-inhibited) conditions (157, 158, 162–165, 170, 186–200). Succinate (157, 158, 163–165, 186–196, 200), glycerol-1-phosphate (162, 170, 194, 196–199), or capronate (162, 196) was the added substrate. The parameters measured were the same as in the nonrespiring systems; but, in addition, the oxygen uptake was recorded; thus the influence of the phosphorylation potential on respiratory control was ascertained.

Systems of the second type involve, in principle, the oxidative generation of high-energy intermediates at one coupling site of the respiratory chain, and their utilization for reversing electron transport at another coupling site. In practice, the systems studied have involved (*a*) generation of high-energy intermediates at Coupling Sites II and III, and their utilization for reversing Coupling Site I—e.g., reduction of NAD^+ (without or with an NADH-oxidizing metabolite such as acetoacetate or α-ketoglutarate+NH_4^+) by succinate or glycerol-1-phosphate, coupled to aerobic oxidation of these substrates (74, 82, 162, 163, 166–169, 188, 194, 196, 198, 201–213); (*b*) generation of high-energy intermediates at Coupling Site II, and their utilization for reversing Coupling Site I—e.g., reduction of NAD^+ (acetoacetate) by succinate, coupled to the oxidation of succinate by ferricyanide (74, 211); (*c*) generation of high-energy intermediates at Coupling Site III, and their utilization for reversing Coupling Sites I and II—e.g., reduction of NAD^+ (α-ketoglutarate$+NH_4^+$) by ascorbate+TMPD, coupled to the aerobic oxidation of ascorbate+TMPD (212, 214–220); (*d*) generation of high-energy intermediates at Coupling Site III, and their utilization for reversing Coupling Site I—e.g., reduction of NAD^+ (α-ketoglutarate$+NH_4^+$) by succinate coupled to the aerobic oxidation of ascorbate+TMPD (aerobic oxidation of succinate blocked by antimycin A) (168, 214–216, 219, 221). These reactions have so far been observed only with intact mitochondria, but there is evidence that utilization of high-energy intermediates from the respiratory chain for purposes other than reversal of energy coupling, viz., NADH-linked $NADP^+$ reduction, can also take place in submitochondrial particles (222–225), and even in nonphosphorylating preparations (226); these systems will be considered later.

PATHWAY OF ELECTRON TRANSFER

Most of the current information relating to the mechanism of energy-dependent reversal of electron transport comes from studies of the succinate-linked reduction of NAD^+. Although this reaction has now been observed for over seven years (186), the concept that it involves a reversal of electron transport through the respiratory chain has only recently received general acceptance. The possibility that the observed rapid and extensive reduction of intramitochondrial NAD^+ in the presence of succinate, despite its well-recognized sensitivity to amobarbital and to uncoupling agents, might not involve succinate dehydrogenase and the respiratory chain, but might proceed via endogenous substrates or via malate (derived from succinate) and malate dehydrogenase, appeared to constitute a serious problem. However, the introduction of acetoacetate to reoxidize continuously the NADH arising from the succinate-linked NAD^+ reduction in rat liver mitochondria (74), or of substantial amounts of NAD^+ as the hydrogen acceptor for succinate oxidation in submitochondrial particles (175), conclusively eliminated endogenous substrate as the source of the reducing equivalents. Furthermore, the failure of malate to replace succinate in these systems, as well as the later demonstration (166) of a stoichiometric relationship between succinate consumed and malate formed during the succinate-linked acetoacetate reduction, also excluded the possibility that dismutation via malate and malate dehydrogenase could be the reaction pathway involved.

Criticism raised against the concept of the energy-dependent succinate-linked NAD^+ reduction, based on a lack of sensitivity to amobarbital and DNP in tissue homogenates (227–229), could be eliminated by experiments in which the effect of the cell sap on the mitochondrial succinate-linked acetoacetate reduction was investigated (207). Although lack of inhibition of succinate-linked acetoacetate reduction by amobarbital and DNP also has been reported with isolated mitochondria (230), this has later been shown by Azzone et al. (209) to be probably connected with a deficient capacity of the preparations used to maintain respiratory control. The importance of the structural integrity of the mitochondria in determining which path—

the energy-linked one or that over malate—is preferred in the succinate-linked NAD^+ reduction has also been emphasized in the studies of Avi-Dor et al. (202).

While most workers in this field now seem to agree that the succinate-linked NAD^+ reduction involves succinate dehydrogenase and NADH dehydrogenase, there is still considerable uncertainty concerning the electron transfer pathway between the two flavoproteins. More specifically, the question is whether the interaction between the two flavoenzymes is a direct one or whether it involves additional carriers, notably coenzyme Q and cytochrome-*b*. The participation of cytochrome-*b* was implicated originally by Chance & Hollunger (187) from their finding that antimycin A inhibited succinate-linked NAD^+ reduction. Although the site of action of antimycin A is generally thought to be between cytochromes-*b* and -c_1, i.e., outside the electron path here concerned, it was visualized that antimycin A might combine with reduced cytochrome-*b* (cf. 9), thus blocking its reoxidation by both cytochrome-c_1 and NADH dehydrogenase. However, subsequent studies of Löw et al. (175) with beef heart electron transport particles revealed that antimycin A in amounts sufficient to block succinoxidase completely was entirely without effect on the rate of succinate-linked NAD^+ reduction. Only when antimycin A was used in quantities 10 to 50 times higher than that required for blocking succinoxidase (175–177)—or ascorbate-TMPD-linked NAD^+ reduction (184)—did a partial inhibition of the succinate-linked NAD^+ reduction occur. Essentially similar results were reported by Packer & Denton (221), by Tager & Slater (167), and by Sanadi et al. (182, 183). Likewise, Ernster and associates have presented evidence for the insensitivity of the succinate-linked NAD^+ reduction to low levels of antimycin A (74, 211), and have discussed (210) their results in relation to those of Chance & Hollunger (190). In addition, the data of Estabrook (57) show that, in liver mitochondria, the molar amount of antimycin A needed to block succinoxidase is about one third of the amount of cytochrome-*b* present; these data also suggest that antimycin A may act primarily on the nonheme iron in the cytochrome-*b*-c_1 complex, in accordance with earlier observations of Tappel (60). If a chelation of cytochrome-*b*-c_1-linked nonheme iron were the primary mode of action of antimycin A, then massive amounts of antimycin A might interfere also with the nonheme iron involved in the NADH and succinate dehydrogenases, and thereby suppress succinate-linked NAD^+ reduction.

Chance (157) reported that addition of ATP to nonrespiring (Na_2S-inhibited) mitochondria in the presence of succinate resulted in a reduction of NAD^+ and cytochrome-*b*, while cytochrome-*c* and cytochrome-*a* were oxidized. Similar findings were reported by Hommes (172–174) with submitochondrial particles. These findings were taken as evidence that cytochrome-*b* is involved as an electron carrier between succinate dehydrogenase and NADH dehydrogenase in the succinate-linked NAD^+ reduction. A more general interpretation of these data is that, under the prevailing conditions, the total rate of reduction of cytochrome-*b* by succinate dehydrogenase and by cytochrome-*c* was higher than the rate of its oxidation by NAD+; the observed net effect thus left the question open as to the location of cytochrome-*b* in the respiratory chain relative to the site of entrance of electrons from succinate dehydrogenase. Activation of succinate oxidation by ATP may also be an important factor in bringing about cytochrome-*b* reduction, as indicated by earlier data of Azzone et al. (195).

In their studies of the succinate-linked acetoacetate reduction in rat liver mitochondria, Ernster et al. (210) found a depression of acetoacetate reduction and a simultaneous increase in oxygen uptake when a catalytic amount of vitamin K_3 was added. Evidence was presented that vitamin K_3 acted via the dicoumarol-sensitive flavoprotein, DT diaphorase (73). It had been shown previously that DT diaphorase, in the presence of vitamin K_3, transfers electrons from pyridine nucleotide to cytochrome-*b* (70). If the electrons from succinate dehydrogenase would enter the respiratory chain at the level of cytochrome-*b*, then transfer of electrons from NADH to cytochrome-*b* by way of DT diaphorase and vitamin K_3 would not be expected to compete with the succinate-linked acetoacetate reduction, since any electrons trapped from NADH would re-enter the respiratory chain at the same site they entered when coming from succinate. Hence, it was concluded (210) that the entrance of electrons from succinate dehydrogenase occurs on the substrate-side of cytochrome-*b* and, consequently, that cytochrome-*b* is not on the electron path from succinate to NAD+.

The final decision as to whether cytochrome-*b* is involved as an electron carrier in the succinate-linked NAD+ reduction will greatly depend on the settlement of the role of coenzyme Q in this process, and in the respiratory chain in general. Clearly, if coenzyme Q proves to be the merging point of the succinate and NADH oxidase systems then such a role of cytochrome-*b* is automatically eliminated:

$$\begin{tikzcd}
\text{succ} \arrow[r, leftrightarrow] & \text{Fp}_s \arrow[dr] & & & \\
& & \text{CoQ} \arrow[r, leftrightarrow] & \text{cyt} - b \arrow[r, dotted] & O_2 \\
\text{NADH} \arrow[r, leftrightarrow] & \text{Fp}_d \arrow[ur] & & &
\end{tikzcd}$$

Studies by Chance et al. (163, 170) first indicated an involvement of coenzyme Q in the succinate-linked NAD+ reduction, and Penefsky (219, 220) presented evidence later for the involvement of coenzyme Q in the ascorbate-TMPD-linked reduction of NAD+. These results are compatible with such a role of coenzyme Q. A complication, however, may be the existence of two separate coenzyme Q "compartments," one for the NADH and one for the succinate branch of the respiratory chain with cytochrome-*b* as the joining point:

$$\begin{tikzcd}
\text{succ} \arrow[r, leftrightarrow] & \text{Fp}_s \arrow[r, leftrightarrow] & \text{CoQ} \arrow[dr] & & \\
& & & \text{cyt} - b \arrow[r, dotted] & O_2 \\
\text{NADH} \arrow[r, leftrightarrow] & \text{Fp}_d \arrow[r, leftrightarrow] & \text{CoQ} \arrow[ur] & &
\end{tikzcd}$$

Indications of a compartmentation of coenzyme Q come from recent studies of Szarkowska & Klingenberg (231), using an improved and thoroughly documented analytical procedure for measuring the oxidation-reduction changes in coenzyme Q in intact mitochondria under various metabolic conditions. They found that CoQ was reduced extensively only by succinate, and not by NAD^+-linked substrates and, furthermore, that the degree of reduction increases with the tightness of the coupling of oxidative phosphorylation. These interesting results thus are indicative of an energy-dependent reduction of coenzyme Q by succinate, analogous to that of NAD^+, and suggest that the requirement for energy in the succinate-linked NAD^+ reduction may extend over several electron transfer steps; evidence for an energy-dependent reduction of a flavoprotein by succinate has earlier been presented by Klingenberg & Bücher (197). Whether or not the energy-dependent electron transfer between the different pools of coenzyme Q involves cytochrome-*b* cannot be decided from the available information. Antimycin A does not seem to inhibit this transfer (231).

The existence of more than two compartments of coenzyme Q is also a pertinent possibility, in view of the occurrence in mitochondria of flavin dehydrogenases other than succinate and NADH dehydrogenase, such as glycerol-1-phosphate, choline, and various fatty acid dehydrogenases. Energy-dependent transfer of hydrogen among these, via coenzyme Q, would be analogous to the energy-dependent hydrogen transfer via NAD^+ and $NADP^+$ among various pyridine nucleotide-linked dehydrogenases.

Azzone & Ernster (232–234) have previously demonstrated a requirement for ATP in succinate oxidation by DNP- (or dicoumarol-) and arsenate-pretreated rat liver mitochondria. The development of an ATP requirement during the pretreatment appeared to involve oxaloacetate formed from endogenous substrate, since it could be prevented by agents that prevent oxaloacetate accumulation; however, once it had occurred, it could not be reversed by removing the oxaloacetate. Azzone & Ernster visualized that oxaloacetate acted by depleting the mitochondria of high-energy phosphate (by way of the oxaloacetate carboxylase reaction), which was needed for the transfer of electrons from succinate dehydrogenase to the respiratory chain. They postulated that the oxidation of succinate by the cytochrome system and the reduction of NAD^+ by succinate involved a common high-energy intermediate at the level of either the NADH dehydrogenase flavin or a quinone. Later, however, Slater & Kemp (235) pointed out that the activation of succinate oxidation by ATP is insensitive to oligomycin, whereas the ATP-supported succinate-linked NAD^+ reduction is oligomycin-sensitive. More recently, Åkerblom et al. (236) reinvestigated the effects of ATP and oxaloacetate, using NAD^+-depleted (phosphate-aged) rat liver mitochondria and added oxaloacetate. Low concentrations of oxaloacetate inhibited succinate oxidation, and subsequent removal of the oxaloacetate (by way of transaminase or malate dehydrogenase) did not relieve the inhibition unless ATP was added. On the other hand, complete removal of the oxaloacetate was not necessary for ATP to act, and it was found that the threshold concentration of oxaloacetate, the effect of which ATP was still able to overcome, could be increased dramatically, up to 10 m*M*, by the addition of catalytic amounts of NAD^+ and of a saturated C_6- to C_{12}-fatty acid. Similar effects were observed when ferricyanide, in the presence of antimycin A, was used as the electron acceptor for succinate oxidation, and also with mitochondria from rat skeletal muscle (237). The results were interpreted to indicate that mitochondria contain, and can produce, an oxidized fatty acid derivative which regulates the sensitivity of succinate dehydrogenase towards oxaloacetate. These findings may lead to an explanation of the widely recognized phenomenon (238–250) that ATP and the metabolic state of the mitochondria are critical factors in determining the rate of succinate oxidation in relation to the prevailing level of oxaloacetate.

PATHWAY OF ENERGY TRANSFER

As indicated initially in this section, energy-dependent reversal of electron transport in the respiratory chain can proceed both by way of a reversal of oxidative phosphorylation and by direct transfer of energy between the energy-coupling sites of the respiratory chain.

Studies of the reversal of oxidative phosphorylation, in particular by Chance (158) and by Klingenberg & Schollmeyer (159–162, 198), have led to important generalizations concerning the thermodynamic relationship between the respiratory chain and the components of the phosphorylating system, P_i, ADP, and ATP. The quantitative aspects of this relationship will be considered later (cf. section on stoichiometry). Qualitatively it implies a dynamic equilibrium between the redox pattern of the respiratory chain and the so-called "phosphate potential," i.e., the $[ATP]/[ADP][P_i]$ ratio. Thus, it has been shown that in nonrespiring mitochondria (158–162) the extent to which electron transfer can be reversed from the cytochrome or flavin region to NAD^+ is strictly dependent on the phosphate potential rather than on the actual level of ATP. In respiring mitochondria (162, 198), an influence of the phosphate potential on the state of respiratory control could also be demonstrated, and it was pointed out (162) that the respiratory control may be "an important physiological example of the reversibility of oxidative phosphorylation."

It has been reported (159, 165, 166) that Mg^{++} suppresses, and EDTA stimulates, the ATP-supported reversal of electron transport in mitochondria. The mechanism of these effects is not clear. Most interesting is the fact that the inhibitory effect of Mg^{++} is only observed with intact mitochondria and submitochondrial particles prepared with digitonin, not with submitochondrial particles produced by sonic oscillation. In fact, externally added Mg^{++} is obligatory in the latter case (176). The loss of Mg^{++} sensitivity is paralleled by a loss of sensitivity of the energy transfer reactions to atractyloside and also by a loss of the strict specificity of the energy transfer system for adenine nucleotides (177).

The inhibitory effect of Mg^{++} on the reversal of oxidative phosphorylation in intact mitochondria furnishes a reasonable explanation for the well-known phenomenon that ATP can be accumulated during oxidative phosphorylation as long as ADP and P_i are present, and the rate is independent of the [ATP]/[ADP][P_i] ratio. Thug Mg^{++} appears to maintain the respiratory carriers and the energy-coupling mechanism in a functional state independent of the actual phosphate potential. The mechanism by which Mg^{++}brings about this effect can certainly not be a simple chelation of ATP, since the inhibition occurs also when the concentration of ATP exceeds that of Mg^{++}; furthermore, a similar inhibition should also occur in the case of submitochondrial particles. A pertinent possibility is that in the reaction of ATP with the transphosphorylating enzyme, an enzyme-Mg complex with ADP is formed which is firmly associated in the case of intact mitochondria (or digitonin particles), and thus ADP cannot be released unless it is reconverted into ATP, but is only loosely bound in the case of sonic particles. This would explain the fact that Mg^{++} inhibits the reversal of oxidative phosphorylation, but not the forward process or the P_i-ATP exchange reaction (cf. 208).

The concept of direct transfer of energy between energy-coupling sites of the respiratory chain originates from the observation (211) that oligomycin does not inhibit the energy-dependent reduction of acetoacetate by succinate, coupled to the aerobic oxidation of succinate. When the succinate-linked acetoacetate reduction was suppressed by the addition of phosphate and phosphate acceptor, oligomycin, in an amount sufficient to inhibit ATP formation, restored the reaction. Hence it was concluded (211) that the transfer of energy from high-energy intermediates formed during the aerobic oxidation of succinate to the form in which it was utilized for the succinate-linked reduction of NAD^+ did not involve ATP, but took place directly by a pathway operating above the oligomycin-sensitive site of the energy transfer system. A closer definition of this site became later possible because of two findings: (*a*) that oligomycin inhibits the mitochondrial $O^{18}P_i$-H_2O exchange reaction (117); and (*b*) that arsenate, unlike DNP, does not relieve the inhibition of tightly coupled respiration by oligomycin, i.e., that oligomycin acts below the uncoupling site of DNP but above that of arsenate (116, 119). Both of these lines of evidence strongly suggested that oligomycin blocks the interaction between a nonphosphorylated high-energy intermediate and P_i, thus inhibiting the formation of the phosphorylated high-energy intermediate involved in the formation of ATP. From this conclusion it followed that the transfer of energy in the succinate-linked acetoacetate reduction, driven by the aerobically generated high-energy compounds, in all likelihood took place without the participation of phosphorylated intermediates; or, in more general terms, that the generation, transfer, and utilization of high-energy intermediates in the mitochondria can proceed without the involvement of any phosphorylation proper. Direct evidence for this conclusion was subsequently presented by Ernster (211) by showing that succinate-linked acetoacetate reduction proceeded at unchanged rate in mitochondria whose endogenous P_i had been depleted in the presence of substrate, ADP, hexokinase, and glucose, and whose respiration was limited by lack of P_i. These findings and conclusions were shortly confirmed by Snoswell (201) and later also by others (166, 167, 216). Moreover, both Snoswell (201) and Ernster (208) demonstrated that the succinate-linked NAD$^+$ reduction driven by aerobically generated high-energy intermediates was inhibited by arsenate, and that this inhibition, in contrast to that by DNP, was relieved by oligomycin. This agreed with earlier conclusions of Estabrook (119) and of Huijing & Slater (116) regarding the site of action of oligomycin in relation to those of arsenate and DNP. The recognition of the direct utilization of nonphosphorylated high-energy intermediates for energy-requiring processes in mitochondria has important implications for the interpretation of mechanisms involved in respiratory energy coupling and in various energy-linked functions of the mitochondria, as will be discussed later.

STOICHIOMETRY

Several attempts have been made to determine the stoichiometry of the succinate-linked NAD$^+$ reduction in terms of number of high-energy bonds expended per molecule of NAD$^+$ reduced ($\sim$/NADH ratio). From measurements of the amount of ADP formed from ATP during succinate-linked NAD$^+$ reduction in pigeon heart mitochondria, Chance (200) first arrived at $\sim$/NADH ratios ranging between 1.8 and 5.4, but pointed out that no correction was made for ATP used in other reactions. Consequently, these values may be overestimates (cf. 161, 169, 203, 210). More recently, Chance et al. (193) correlated the succinate-linked NAD$^+$ reduction with simultaneous recording of the ATPase activity by means of a glass electrode. Calculated on the basis of the total ATPase activity, the $\sim$/NADH ratio was 2.8; when only the increase in ATPase activity, attributable to the addition of succinate, was used as a basis, the resulting ratio was 1.4. In this calculation it was assumed: (*a*) that the ATP hydrolysis caused by succinate-linked NAD$^+$ reduction was additive to, and noncompetitive with, the basal ATPase; and (*b*) that no rephosphorylation of ADP to ATP, connected with the aerobic oxidation of succinate, took place under the prevailing conditions (192). If either of these conditions was not fulfilled, the calculation would lead to an underestimate of the $\sim$/NADH ratio.

In another attempt to estimate the $\sim$/NADH ratio, Chance & Hollunger (158, 187) recorded the oxidation and reduction changes in cytochrome-*a* occurring in partially azide-inhibited mitochondria upon the addition of succinate. In this system, succinate causes a reduction of NAD$^+$; and, simultaneously, cytochrome-*a* goes through an oxidation-reduction cycle, first becoming more reduced before returning to its original level. This oxidation-reduction cycle, which is due to utilization of high-energy bonds, is also found after ADP addition. The area under the curve relating cytochrome-*a* reduction with time is a measure of the amount of electron transfer along the respiratory chain to oxygen, i.e., of the oxygen uptake, and is proportional to the amount of ADP added. Thus, it may be possible to calculate, from the area under the curve obtained when succinate is added, the amount of high-energy bonds utilized. In this way a ~/NADH ratio of 2.5 was obtained. When corrected for the utilization of high-energy bonds for reactions other than the reduction of NAD^+ (the latter being eliminated by the addition of amobarbital), the value became 1.7. This method for calculating the ~/NADH ratio involves the assumption that phosphorylation of ADP and the succinate-linked NAD^+ reduction are equally efficient in utilizing high-energy intermediates from the respiratory chain. To what extent this is the case seems to depend on the stability of the high-energy intermediates (i.e., the "tightness" of the energy-coupling system) and on the relative rates of the two energy-utilizing reactions; if the rate of the succinate-linked NAD^+ reduction is slower than the phosphorylation of ADP, it will compete less efficiently with a hydrolytic breakdown of high-energy intermediates than will the phosphorylation, and the obtained ~/NADH ratio will thus be an overestimate (cf. 210).

In the experiments described above, succinate-linked NAD^+ reduction was measured with intramitochondrial NAD^+ as the terminal hydrogen acceptor. Under these conditions, the observed increase in NADH concentration is a measure of an increase in steady state rather than that of the total amount of NAD^+ reduced (cf. 161, 169). Estimates of the ~/NADH ratio have also been performed under conditions where the mitochondrial NADH arising from succinate-linked NAD^+ reduction was continuously reoxidized by an added hydrogen acceptor. Ernster (74, 211) calculated the ~/NADH ratio from the respiratory increase caused by acetoacetate in rat liver mitochondria oxidizing succinate in the absence of phosphate acceptor. It was concluded that the ratio was 1. This conclusion rests on the assumptions that: (*a*) the aerobic oxidation of succinate is connected with the generation of two high-energy bonds per atom of oxygen consumed; (*b*) both of these can be utilized for succinate-linked NAD^+ reduction; and (*c*) only the respiratory increase and not the basic, loosely coupled respiration of the system contributes high-energy bonds to the succinate-linked NAD^+ reduction. In order to eliminate the need for these assumptions, in a subsequent study (210), the amount of acetoacetate reduced was compared with the phosphate uptake obtained in a parallel system where a corresponding respiratory increase was induced by the addition of ADP, glucose, and a limiting amount of hexokinase. From these measurements, a P_i/AcAc ratio of 1.19 was obtained, and thus the previous estimate of one high-energy bond equivalent expended per molecule of NAD^+ reduced by succinate was essentially confirmed. Independently, Slater & Tager (169) arrived at the same conclusion from studies of succinate-linked aminative reduction of α-ketoglutarate. This reaction [in contrast to the less powerful acetoacetate reduction (cf. 74)] efficiently competes with the phosphorylating system for the high-energy bonds derived from the respiratory chain, thus giving rise to a substantial decrease of the P/O ratio. Slater & Tager (169) calculated the

~/NADH ratio from the difference in phosphate uptake observed during succinate oxidation in the absence and presence of α-ketoglutarate+NH_4^+. In confirmation of the conclusions of Ernster et al. (74, 210, 211), they arrived at values close to one. A ~/NADH ratio of near unity was also obtained when the aerobic oxidation of ascorbate+TMPD provided the energy for the succinate-linked NAD^+ reduction, as measured in the presence of antimycin A. From the collected evidence we conclude that the succinate-linked NAD^+ reduction requires the expenditure of one high-energy bond per molecule of NAD^+ reduced by succinate:

$$\text{Succinate} + \text{NAD}^+ + \text{I} \sim \text{X} \rightleftharpoons \text{Fumarate} + \text{NADH} + \text{H}^+ + \text{I} + \text{X}$$

where I~X can be generated either by ATP or by either of the two terminal coupling sites of the respiratory chain. The conclusion that both Coupling Sites II and III can provide energy for the reaction has been evident from the findings that succinate-linked NAD^+ reduction can occur with either the aerobic oxidation of ascorbate+TMPD (216) or the oxidation of succinate by ferricyanide (74, 211) as the source of energy, and is further substantiated by the equal ~/NADH ratios obtained by Slater & Tager (169) for succinate and ascorbate+TMPD as the energy-yielding substrates. The fact that only one high-energy bond is required for the reversal of electron transport through Coupling Site I of the respiratory chain appears to eliminate NADH~I as an intermediate in the NAD-flavin-linked respiratory chain phosphorylation; for a discussion of this problem, see Ernster, Chance & Klingenberg (251).

Attempts have also been made to determine the ~/NADH ratio for reversal of electron transport over more than one coupling site of the respiratory chain. Tager et al. (216) found that ascorbate+TMPD was a relatively inefficient hydrogen donor for glutamate synthesis in rat liver mitochondria, and attempts by Slater & Tager (169) to estimate the ~/NADH ratio for the ascorbate+TMPD-linked glutamate synthesis gave variable results. Löw et al. (177) compared the increase in ATP hydrolysis accompanying the succinate-linked and the ascorbate+TMPD-linked NAD^+ reduction in submitochondrial particles from beef heart and found that this increase was twice as great in the case of ascorbate+TMPD as in the case of succinate. Thus, accepting the value of one as the ~/NADH ratio for the succinate-linked NAD^+ reduction, the ~/NADH ratio for the reduction of NAD^+ by ascorbate+TMPD (i.e., by reduced cytochrome-*c*) appears to be two.

Chance & Hollunger (158) and Klingenberg (161) have determined the $[ATP]/[ADP][P_i]$ ratio that is required to cause half-oxidation of cytochrome-*c* in nonrespiring (Na_2S- or KCN-inhibited) mitochondria from pigeon heart and rat liver, respectively. Chance & Hollunger (158) did not use an electron acceptor to trap electrons from NADH, but an endogenous acceptor of unknown nature—"an infinite electron sink"—was present, as revealed by the fact that the sum of oxidation of cytochrome-*a*, -*c*, and -*b*, and of flavin, was greater than the reduction of pyridine nucleotide. The

[ATP]/[ADP][P_i] ratio giving half-oxidation of cytochrome-*c* was 10^4; from this the value of $\Delta F_0 = -15$ kcal ($\Delta E_0 = 0.3$ V) for the oxidation of reduced cytochrome-*c* by the unknown electron acceptor was computed. Assuming that the unknown acceptor operates via NAD$^+$, this is a surprisingly low value, about one half of that expected from the difference in redox potential between cytochrome-*c* and NAD$^+$, and indicates that the "electron sink" would be at the level of flavoprotein. On the other hand, in Klingenberg's system, oxaloacetate was added as electron acceptor, which ought to act via NAD$^+$, and still, the [ATP]/[ADP][P_i] ratio required for half-oxidation of cytochrome-*c* was 10^3, i.e., even somewhat lower than that found by Chance & Hollunger (158); the corresponding $-\Delta F_0$ value is 12.4 kcal ($\Delta E = 0.270$ V). The interpretation given by Klingenberg (161) is that this value does not represent the actual change of free energy for the whole span cytochrome-*c*→NAD$^+$, but only for the hydrolysis of the high-energy intermediate of the cytochrome-*b*-*c* energy coupling site, and he assumes that this intermediate is an energized form of reduced cytochrome-*c*. A better understanding of the actual chemical mechanism, and in particular of the rôle of coenzyme Q (which might be the "electron sink"), will be necessary in order to evaluate the implications of these intriguing results properly.

Energy-dependent reduction of NAD$^+$ by reduced cytochrome-*a* has been observed by Chance (163) and by Klingenberg & Schollmeyer et al. (159, 160, 162, 196, 198), but no data regarding the ~/NADH ratio for this reaction are yet available. It is also uncertain whether the reaction involves a reversal of electron transport over all three coupling sites of the respiratory chain or only over Sites I and II. The decision of this question is dependent on whether Coupling Site III will prove to occur between cytochrome-*c* and cytochrome-*a*, as postulated by Chance & Williams (9) on the basis of kinetic evidence, or between cytochrome-*a* and oxygen, as envisaged by Slater (252) from thermodynamic considerations.

ENERGY-LINKED HYDROGEN TRANSFER AT THE PYRIDINE NUCLEOTIDE LEVEL

It has been known for some time that mitochondria are capable of utilizing reduced pyridine nucleotides for various types of reductive reactions. Such reactions have mostly been studied by coupling them to a pyridine nucleotide-linked oxidation. The classical example of this type of coupled reaction, often called pyridine nucleotide-linked dismutation, is that described by Krebs & Cohen (253) in 1939 in kidney cortex and heart muscle. It involves the reduction of α-ketoglutarate, in the presence of NH_4^+, to glutamate, by NADH generated through the oxidation of another molecule of α-ketoglutarate. Later Hunter & Hixon (254) studied this reaction in mitochondrial preparations from rat liver and used it for the demonstration of the phosphorylation coupled to the oxidation of α-ketoglutarate by NAD$^+$.

Another type of pyridine nucleotide-linked hydrogen transfer in mitochondria is the pyridine nucleotide transhydrogenase reaction described in

1953 by Kaplan et al. (255). It consists of a reversible transfer of hydrogen between NADH and NADPH. The reaction, which has been investigated mainly in digitonin extracts of mitochondria, has an equilibrium constant of near unity, in accordance with the closely similar redox potentials of the two pyridine nucleotides. Yet it has been noticed that the reaction proceeds more rapidly when starting from NADPH and NAD$^+$ than in the reverse direction.

Recently both the above types of reaction have acquired renewed interest since it has been recognized that both pyridine nucleotide-linked dismutation and pyridine nucleotide transhydrogenation can occur in mitochondria in an energy-dependent manner. Moreover, the two types of reaction might have a common denominator in the form of an energy-dependent interaction of NADH and NADP$^+$ to yield NAD$^+$ and NADPH.

Pyridine Nucleotide Transhydrogenase

Indications of the existence of an energy-dependent reduction of NADP$^+$ date back to 1954 when Krebs (256) observed that DNP markedly suppressed the aerobic formation of malate from pyruvate and CO$_2$ in pigeon liver homogenate. Krebs suggested that DNP prevented the formation of ATP and that ATP shifted the equilibrium of a reaction involving NADP$^+$ and NADPH in favor of NADPH, the latter being necessary for the carboxylative reduction of pyruvate to malate. He envisaged a mechanism involving an ATP-dependent reduction of NADP$^+$ by a reduced flavoprotein.

Klingenberg & Slenczka (204) found in 1959 that incubation of rat liver mitochondria with NAD$^+$-specific substrates or succinate in the absence of phosphate acceptor resulted in a rapid and almost complete reduction of the mitochondrial NADP$^+$. Addition of ADP and of DNP abolished the NADP$^+$ reduction, the second more efficiently. These and related findings led Klingenberg and associates (162, 194, 204) to postulate that the equilibrium of the pyridine nucleotide transhydrogenase reaction in mitochondria, earlier described by Kaplan et al. (255), is controlled by the phosphate potential so that a high ratio of [ATP]/[ADP][P$_i$] may bring about a considerable (10- to 15-fold) shift of the equilibrium towards the formation of NADPH. Similar observations were later reported by Estabrook & Nissley (257).

In 1963 Danielson & Ernster (222–224) demonstrated an energy-dependent conversion of NADH and NADP$^+$ into NAD$^+$ and NADPH in phosphorylating electron transport particles derived by sonication of mitochondria from rat liver and beef heart. The energy for the reaction could be supplied either by added ATP, or by the aerobic oxidation of NADH or succinate. Presence of Mg^{++} was obligatory for the former, but not for the latter system. Oligomycin inhibited the ATP-supported reaction, whereas it did not affect, or even slightly stimulated, the reaction as proceeding at the expense of aerobically generated high-energy intermediates. DNP inhibited the reaction in both cases, but was more efficient with aerobically generated high-energy intermediates than with ATP as the source of energy. In the

ATP-supported system the reaction could be coupled to the succinate-linked reduction of NAD^+. ITP and GTP, and to a lesser extent UTP and CTP, were able to replace ATP as the source of energy for the pyridine nucleotide interaction, and these reactions paralleled the corresponding nucleoside triphosphatase activities in terms of both relative velocities and oligomycin sensitivity. The energy requirement of the reaction was measured by comparing the amount of P_i liberated from ATP with the amount of NADPH formed. The P_i/NADPH ratio varied from 15 to 1.4, decreasing with the concentration of ATP employed; a P_i/NADPH ratio of one was estimated by extrapolation to infinitely low ATP concentration. It was also shown that the equilibrium of the energy-dependent reaction is strongly shifted towards the formation of NAD^+ and NADPH; recently Lee & Ernster (225) made a quantitative estimate of the equilibrium shift and found an equilibrium constant ($K = [NAD^+] \cdot [NADPH]/[NADH] \cdot [NADP^+]$) for the energy-dependent reaction of 480 as compared with the value of 0.79 obtained for the nonenergy-dependent transhydrogenase reaction. The rate of the ATP-supported reaction was unaffected by ADP and P_i; this was in contrast to the succinate-linked NAD^+ reaction, whose rate was markedly influenced by the phosphate potential. On the basis of these results, the following equation was proposed for the energy-dependent transhydrogenase reaction:

$$NADH + NADP^+ + {\sim}X \rightarrow NAD^+ + NADPH + X$$

where ${\sim}X$ can be generated either by ATP:

$$ATP + X \rightleftharpoons ADP + P_i + {\sim}X$$

or by the respiratory chain:

$$AH_2 + B + X \rightleftharpoons A + BH_2 + {\sim}X$$

(A and B denoting respiratory carriers, and ${\sim}X$ a nonphosphorylated high-energy intermediate).

Recently Lee et al. (226) have demonstrated energy-dependent transhydrogenase reaction with various types of nonphosphorylating particles. These were not able, or only slightly so, to utilize ATP for NADH-linked $NADP^+$ reduction, but exhibited an appreciable transhydrogenase activity with succinate oxidation as the source of energy. Further treatment of the nonphosphorylating pzrticles with 2.5 *M* urea also led to an 80 percent loss of the nonenergy-dependent transhydrogenase activity (measured as NADPH-linked NAD^+ reduction), without any significant decrease in the activity of the energy-dependent, succinoxidase-supported, NADH-linked $NADP^+$ reduction. These results have revealed: (*a*) that nonphosphorylating electron transport particles can perform respiratory energy-coupling even though the phosphorylating ability as such is lost; and (*b*) that the nonenergy-dependent transhydrogenase is not rate limiting for—and possibly unrelated to—the energy-dependent reaction.

Hommes & Estabrook (258) shortly confirmed the initial findings of

Danielson & Ernster (222) and further investigated the ATP-supported NADH-linked NADP+ reduction in relation to the nonenergy-dependent transhydrogenase reaction as well as to the succinate-linked NAD+ reduction. They concluded (179, 258, 259) that the energy- and nonenergy-dependent reactions are catalyzed by the same enzyme and, furthermore, that the energy-dependent transhydrogenase reaction and the succinate-linked NAD+ reduction involve a common high-energy intermediate at the NAD+ level. These conclusions were based on three lines of evidence: (*a*) Mg++ inhibits the nonenergy-dependent transhydrogenase reaction at the same time as it stimulates both the ATP-dependent transhydrogenase reaction and the ATP-dependent succinate-linked NAD+ reduction; it was visualized that Mg++ may interact with an NAD+-containing high-energy intermediate, rendering it available or unavailable for hydrogen transfer depending on whether or not ATP is present; (*b*) triiodothyronine inhibits the two types of transhydrogenase reaction and the succinate-linked NAD+ reduction with the same efficiency; (*c*) all three reactions possess a high activation energy of the order of 20 kcal/mole.

Several facts, however, suggest caution in drawing the above conclusions. NAD+ is substrate of the succinate-linked NAD+ reduction, and product of the NADH-linked NADP+ reduction. It is unlikely, therefore, that the two reactions would involve a common high-energy intermediate at the NAD+ (or NADH) level. Moreover, octylguanidine, which is a specific inhibitor of the NAD+-flavin-linked respiratory-chain phosphorylation (133), and which inhibits succinate-linked NAD+ reduction (174), has no effect on the energy-dependent NADH-linked NADP+ reduction (260). As to the relationship of the two types of transhydrogenase reaction, triiodothyronine inhibits, besides the two types of transhydrogenase and the succinate-linked NAD+ reduction, submitochondrial ATPase (261), purified DT diaphorase (73), and a series of other enzymes (cf. 262). Likewise, activation energy of the order of 20 kcal/mole is a feature common to many enzyme reactions. Mg++ inhibits the nonenergy-dependent reaction in a manner competitive with respect to NAD+ (179), and is without effect on its equilibrium (225). Thus, Mg++ does not seem to act by stabilizing a high-energy intermediate as has been suggested (179, 236). It also neither stimulates nor inhibits the energy-dependent reaction when this is supported by aerobically generated high-energy intermediates (223, 224). Since Mg++ inhibits the nonenergy-dependent transhydrogenase and is at the same time obligatory for the ATP-supported, energy-dependent reaction, the combined effects of Mg++ and ATP may easily give the impression that the two reactions are interrelated rather than simply superimposed. Thus, these findings leave the question open as to the identity of two types of transhydrogenase. The selective inhibition of the nonenergy-dependent reaction by Mg++ suggests the nonidentity of the two enzymes, as does a similar inhibition recently observed with adenine nucleotides (263). In fact, as pointed out by Ernster & Lee (263), it cannot even be taken for granted at present that the energy-dependent reaction really involves a transhydrogenation and not a group transfer (e.g., transfer of the 2-phosphate group from NADP$^+$ to NADH, or exchange of the adenine nucleotide moieties of the two pyridine nucleotides).

McGuire et al. (264) have briefly reported that, in submitochondrial particles from rat liver, 10^{-4} *M* dicoumarol inhibits both the ATP-supported reduction of NADP$^+$ by NADH, and a nonenergy-dependent reduction of acetylpyridine adenine dinucleotide by NADPH. They concluded, in accordance with Hommes & Estabrook (258), that the two transhydrogenase reactions involve similar enzymes. It was further suggested that, since DT diaphorase was present in the particles, and since this enzyme is known to be sensitive to dicoumarol and triiodothyronine, the two transhydrogenase reactions and DT diaphorase may involve a common component. The interpretation of these interesting findings, however, is difficult. NAD$^+$ analogues are not specific reagents for NAD$^+$–NADP$^+$ transhydrogenase but react with diaphorases as well (cf. 2). Also, 10^{-4} *M* dicoumarol is not a selective inhibitor of DT diaphorase (cf. 73), and, in addition, it uncouples respiratory chain-linked phosphorylation (80).

PYRIDINE NUCLEOTIDE-LINKED DISMUTATIONS

The recognition of an energy dependence of certain pyridine nucleotide-linked dismutations in mitochondria arose from the use of α-ketoglutarate +NH_4^+ as a trapping system for reducing equivalents from NADH during the energy-dependent reduction of NAD$^+$ by succinate. Tager (265) and Klingenberg (266) independently observed that the reductive synthesis of glutamate from α-ketoglutarate and NH_4^+ in rat liver mitochondria required energy not only with succinate, but also with malate as the substrate. Their system consisted of rat liver mitochondria incubated in the presence of malate, α-ketoglutarate, NH_4^+, and arsenite, the latter in order to inhibit the oxidation of α-ketoglutarate. In this system malate is oxidized to oxaloacetate with the reduction of mitochondrial NAD$^+$, and the NADH so formed reacts with α-ketoglutarate and NH_4^+ to form glutamate. The glutamate in turn interacts by way of the glutamate-oxaloacetate transaminase with oxaloacetate to yield aspartate and α-ketoglutarate. The sum of glutamate and aspartate formed is a measure of the total amount of glutamate synthesized. Both Tager and Klingenberg found that this reaction was dependent on aerobically generated high-energy intermediates or, if respiration was inhibited, on added ATP. In extensive studies, Tager and associates (168, 169, 213, 215, 216, 267) further demonstrated that the reaction, as supported by aerobically generated high-energy intermediates, in contrast to that supported by ATP, was insensitive to oligomycin, and thus probably utilized nonphosphorylated high-energy intermediates. The latter could be generated by the aerobic oxidation of malate, or, when this was blocked by antimycin A, by the oxidation of ascorbate+TMPD. These properties were similar to those found for the succinate-linked glutamate synthesis.

A difference between the two reactions, however, was found in the energy requirement. A ratio of number of high-energy bonds expended per molecule of glutamate synthesized of approximately one was estimated for the succinate-linked glutamate synthesis, whereas the corresponding value was approximately 0.25 and 0.6, respectively, for the malate-linked glutamate synthesis as supported by the aerobic oxidation of malate and of ascorbate +TMPD. In other words, up to four molecules of glutamate per high-energy bond expended were synthesized in the malate-linked system, in contrast to the one to one stoichiometry of the succinate-linked glutamate synthesis. When isocitrate was used instead of malate as the source of hydrogen, the synthesis of glutamate from α-ketoglutarate+NH_4^+ proceeded rapidly under anaerobic conditions, and its rate was not influenced by added ATP. Both the energy-dependent (succinate- or malate-linked) and the nonenergy-dependent (isocitrate-linked) glutamate synthesis were stimulated by P_i.

Tager & Slater (213) also investigated the α-ketoglutarate-linked glutamate synthesis, i.e., the Krebs-Cohen dismutation, and found, in confirmation of earlier observations of Krebs et al. (268), that α-ketoglutarate was a relatively poor hydrogen donor for glutamate synthesis as compared to the other substrates. Some aspects of this reaction were independently studied by Danielson & Ernster (269). They found that the Krebs-Cohen dismutation in nonrespiring (antimycin A-inhibited) rat liver mitochondria is greatly enhanced by P_i. Addition of 40 mM fluoride [which had been used by Hunter & Hixon (254) in their studies of α-ketoglutarate-linked substrate-level phosphorylation] abolished the stimulating effect of P_i and simultaneously increased the $P/2e$ ratio from about 0.3 to near 1. Similar effects were obtained with DNP and with oligomycin. Arsenate depressed the rate of dismutation to the same level as fluoride, DNP, or oligomycin; but it also abolished the phosphorylation. A fluoride-induced decrease in the rate of α-ketoglutarate disappearance and a concommitant increase in $P/2e$ ratio occurred also with acetoacetate, rather than α-ketoglutarate as the hydrogen acceptor. Danielson & Ernster (269) concluded that both dismutations required energy, which was supplied by the α-ketoglutarate-linked substrate-level phosphorlyation through a fluoride-, DNP-, and oligomycin-sensitive reaction mechanism. Tager (270) has recently found that malonate stimulates the Krebs-Cohen dismutation, but not the α-ketoglutarate-linked acetoacetate reduction, and interpreted this effect in terms of an enhanced production of NADPH for glutamate synthesis. The formation of NADPH in the presence of malonate was thought to proceed through a transcarboxylation of malonyl-CoA (formed from malonate) with α-ketoglutarate, followed by a reduction of the oxalosuccinate so formed by NADH via the NAD^+-specific isocitrate dehydrogenase, and finally an oxidation of the resulting isocitrate by $NADP^+$ via the $NADP^+$-specific isocitrate dehydrogenase. Stimulation of glutamate synthesis by isocitrate had earlier been observed by Adler et al. (271) and by Krebs et al. (268), and stimulation by malonate had been noticed by Klingenberg & Schollmeyer (159). Various aspects of mitochondrial glutamate synthesis have also been studied by Jones & Gutfreund (272), Worcel & Erecinska (273, 274), Alberti & Bartley (275), and Katunuma & Okada (276).

The need for energy in pyridine nucleotide-linked dismutations in mitochondria is a puzzling phenomenon, not predictable on thermodynamic grounds. The interpretations so far suggested involve a compartmentation of the mitochondrial pyridine nucleotides in relation to the individual dehydrogenases. Klingenberg (277) envisages a "dynamic" compartmentation of the pyridine nucleotides and a "fixed" compartmentation of the dehydrogenases. The latter would be located in three compartments, one outside, one inside the cristae, and one within the crista membrane. Movement of pyridine nucleotides from one compartment to another would proceed by active transport and would be governed chiefly by the phosphate potential. Because of their arrangement in the membrane structure, those dehydrogenases located in the crista membrane may be "directional," i.e., they may catalyze preferentially one direction of the reaction. The transhydrogenase would be such an enzyme, and the energy-controlled transhydrogenase reaction may be described, according to this theory, by the equation:

$$\text{NADH-}\mathit{in} + \text{NADP}^{+}\text{-}\mathit{out} \rightarrow \text{NAD}^{+}\text{-}\mathit{out} + \text{NADPH-}\mathit{in}$$

where "*in*" and "*out*" mean the space in- and outside the crista membrane. This interesting theory is similar to, and is an extension of, the "chemiosmotic" theory of oxidative phosphorylation, earlier proposed by Mitchell (278).

A somewhat different interpretation of the need for energy in pyridine nucleotide-linked dismutations has been proposed by Tager (267, cf. also 168). It is based on the earlier suggestion of Klingenberg et al. (204, 266) that glutamate dehydrogenase is NADPH-specific in mitochondria, as well as on evidence presented by Purvis (279, 280) for the existence of two energized forms of pyridine nucleotide, denoted NAD$\sim$I and NADP$\sim$I, which may interact according to the reactions:

$$\begin{array}{rl}
 & \text{NADH} + \text{NADP}\sim\text{I} \rightleftharpoons \text{NADPH} + \text{NAD}\sim\text{I} \\
 & \text{NADP}^{+} + \text{NAD}\sim\text{I} \rightleftharpoons \text{NADP}\sim\text{I} + \text{NAD}^{+} \\
\cline{2-2}
\text{Net:} & \text{NADH} + \text{NADP}^{+} \rightleftharpoons \text{NAD}^{+} + \text{NADPH}
\end{array}$$

Incorporating this "energy-catalyzed" pyridine nucleotide transhydrogenase mechanism into the malate-linked glutamate synthesis, the latter process may be written as (cf. 267):

$$\begin{array}{rl}
 & \text{Malate} + \text{NAD}^{+} \rightleftharpoons \text{Oxaloacetate} + \text{NADH} + \text{H}^{+} \\
 & \text{NADH} + \text{NADP}\sim\text{I} \rightleftharpoons \text{NADPH} + \text{NAD}\sim\text{I} \\
 & \text{NADPH} + \text{NH}_4^{+} + \alpha\text{-ketoglutarate} \rightleftharpoons \text{Glutamate} + \text{NADP}^{+} \\
 & \text{NADP}^{+} + \text{NAD}\sim\text{I} \rightleftharpoons \text{NADP}\sim\text{I} + \text{NAD}^{+} \\
 & \text{Glutamate} + \text{oxaloacetate} \rightleftharpoons \text{Aspartate} + \alpha\text{-ketoglutarate} \\
\cline{2-2}
\text{Net:} & \text{Malate} + \text{NH}_4^{+} \rightleftharpoons \text{Aspartate} + \text{H}^{+}
\end{array}$$

This mechanism accounts for the findings that energy is needed only in hydrogen transfer from an NAD^{+}-linked to an NADP^{+}-linked dehydrogenase (malate→glutamate; α-ketoglutarate→glutamate), but not in that between two NADP$^+$-linked dehydrogenases (isocitrate→glutamate). It remains to be understood why glutamate dehydrogenase behaves in this respect as a NADP$^+$-linked dehydrogenase when the free enzyme is known to react with both NADH and NADPH. It is conceivable, however, that energy is also required in mitochondrial dismutations betwen two NAD$^+$-linked dehydrogenases, possibly through the involvement of NADP$^+$ as an intermediate hydrogen carrier between the two NAD$^+$'s. There is in fact some indication of an energy requirement in a dismutation between two NAD$^+$-specific dehydrogenases [α-ketoglutarate- and β-hydroxybutyrate-dehydrogenase (cf. 269)]. Also the question of energy expenditure will require further consideration. As stated in the equations, the need for energy is only catalytic; the data, however, show a net expenditure of energy, even though less than stoichoimetric. This problem has been discussed by Slater & Tager (169). The possibility of studying the energy-dependent interaction between NADH and NADP$^+$ in submitochondrial particles may open new approaches to the elucidation of this complex phenomenon.

THE OXIDATION OF ISOCITRATE

The much-debated problem as to whether isocitrate is oxidized in the mitochondria by way of the NAD$^+$- or the NADP$^+$-specific isocitrate dehydrogenase [cf. Plaut (281)] has recently undergone an interesting development. Chen & Plaut (282, 283) have investigated in great detail the properties of the NAD$^+$-specific isocitrate dehydrogenase from bovine liver and found that the enzyme is strongly activated by ADP, and inhibited by ATP, NADH, and adenosine diphosphoribose. Goebell & Klingenberg (284, 285) have reported that mitochondria from various organs exhibited a high NAD$^+$-linked isocitrate dehydrogenase activity when the enzyme was extracted and assayed under appropriate conditions; ADP was essential for the assay. In certain tissues, such as rat brain and locust flight-muscle, the activity of the enzyme even exceeded that of the NADP$^+$-specific isocitrate dehydrogenase. Furthermore, the NAD$^+$-linked—but not the NADP$^+$-linked—enzyme was found to be present in a near-constant relationship to the respiratory chain of the different tissues. These results appear to substantiate the earlier conclusion of Ernster & Navazio (286, 287) that the NAD$^+$ -specific isocitrate dehydrogenase is the main pathway of the aerobic oxidation of isocitrate.

Chappel (248) has reported that the aerobic oxidation of isocitrate in rat liver mitochondria is greatly enhanced by malate. He proposed that the NADP$^+$ reduced by isocitrate dehydrogenase is reoxidized by NAD$^+$ via transhydrogenase, and the NADH in turn is reoxidized by coupling with the malate dehydrogenase. In the light of the new information quoted above, it appears possible that NAD$^+$ is directly reduced by isocitrate, after which the NADH interacts with the malate dehydrogenase system as suggested by Chappell (248). This would eliminate the need for a high transhydrogenase activity, which liver mitochondria do not seem to possess (cf. 287).

Further aspects of isocitrate oxidation in mitochondria have been studied by Hawtrey (288), Marthy & Rappoport (289), and Sanwal et al. (290).

RESPIRATORY CHAIN-LINKED ION TRANSPORT

Active uptake of ions into mitochondria has been observed for over a decade and the subject has been reviewed in 1962 by Lehninger (291). During the past two years, studies primarily by Vasington & Murphy (292–294), DeLuca et al. (295–297), Brierley et al. (298–300), Chappell et al. (301, 302), and Lehninger et al. (303–305) have resulted in new important developments concerning the active accumulation of divalent cations and of inorganic phosphate in mitochondria by a process that is directly linked to the energy transfer system of the respiratory chain.

Mitochondria from a variety of tissues are capable of accumulating large amounts of added Ca^{++} (292–296, 299, 300, 303–305), Mg^{++} (297–300), Mn^{++} (301, 302), and Sr^{++} (302), provided that P_i and a supply of high-energy intermediates generated by the respiratory chain are present. Lack of substrate, respiratory inhibitors, and ucoupling agents such as DNP, dicoumarol, or gramicidin, inhibit the ion uptake. On the other hand, the ion uptake is not inhibited by oligomycin, suggesting that ATP and its phosphorylated precursor are not involved in the process, but the energy is supplied directly by nonphosphorylated high-energy intermediates generated by the respiratory chain. The accumulation of Ca^{++}, but not of Mg^{++}, Mn^{++}, and Sr^{++}, requires the presence of ATP, its role probably being to counteract the uncoupling effect of Ca^{++}. ATP can replace, though to a limited extent, the respiratory chain-linked energy-generating system in supporting ion accumulation, and the process then is oligomycin sensitive. P_i is an obligatory component of the systems carrying out active uptake of Ca^{++} and Mg^{++}, and is accumulated in the mitochondria along with the cations. The compound accumulated in the case of Mg^{++} corresponds in its composition to $Mg_3(PO_4)_2$ (298), and in the case of Ca^{++} it corresponds to $[Ca_3(PO_4)_2]_3 \cdot Ca(OH)_2$ (hydroxyapatite) (304, 305). In the case of Mn^{++}, P_i greatly enhances the accumulation of the cation, but its presence is not absolutely necessary. Chappell and associates (302) have shown that the small amount of Mn^{++} that is taken up in the absence of added P_i exceeds the amount of endogenous P_i in the mitochondria by 10- to 20-fold, and thus cannot be accounted for as $Mn_3(PO_4)_2$. This non-P_i-dependent Mn^{++} uptake differed from the P_i-dependent one in that it was both prevented and reversed by DNP, whereas the latter was only prevented but not reversed. From measurements of the enhancement of the proton relaxation rate it appeared that the Mn^{++} so bound was inaccessible to water in the bulk of the medium and was thus probably bound to a hydrophobic target, possibly a phospholipid. Furthermore, when respiration was inhibited by antimycin A after the non-P_i-dependent Mn^{++} uptake had taken place, P_i added immediately afterwards was taken up in a respiration-independent manner. These data were interpreted to indicate that the cation is taken up by an active process and is followed by P_i in a passive manner.

Rossi & Lehninger (304, 305) have estimated the energy requirement for the accumulation of Ca^{++} and P_i, and found it to be one high-energy bond equivalent per mole of P_i accumulated. This ratio was independent of whether three, or only two or one, of the energy-coupling sites of the respiratory chain were operating. This could be shown by measuring the accumulation of Ca^{++} and P_i in the presence of β-hydroxybutyrate, succinate, or ascorbate+TMPD as substrate, respectively, and by comparing the ratio of P_i accumulated to oxygen consumed with the P/O ratios obtained in parallel samples incubated in the absence of Ca^{++} and in the presence of hexokinase and glucose. Similar measurements by Brierley et al. (298) with Mg^{++} and P_i have yielded ratios of molecules of P_i accumulated to pairs of electrons transferred of 0.5 to 0.6. The lower efficiency of the latter system may be related to a release of $Mg_3(PO_4)_2$ from the mitochondria, this salt being relatively more water-soluble than hydroxyapatite. The fact, that in the case of Ca^{++} and P_i accumulation, P_i and not Ca^{++} bears an integral relationship to the number of high-energy bonds expended suggests that P_i is taken up actively and Ca^{++} accompanies it in a passive fashion. A primary role of P_i is also suggested by the fact that P_i is the common counter-anion in the energy-linked uptake of the various divalent cations (except for the slight extent of Mn^{++} uptake taking place without P_i). On the other hand any phosphorylated intermediate involved in this process must differ from that involved in ATP synthesis in that it can be formed in the presence of oligomycin, i.e., that its formation can proceed without oxygen exchange between P_i and H_2O (cf. 117).

The accumulation of divalent cations and P_i in the mitochondria is a means of releasing energy from the respiratory chain in the absence of ADP. This explains earlier observations (306–308), extended in a recent paper by Chance (309), that small amounts of Ca^{++} in the presence of P_i induce a respiratory increase in mitochondria in the controlled state, similar to that induced by ADP.

An important consequence of the precipitation of insoluble phosphates in the mitochondria is that an equivalent amount of protons is released to the medium. Brierley et al. (298) have shown that the pH of the medium declines as P_i is accumulated by heart mitochondria, according to the reaction:

$$3Mg^{++} + 2HPO_4^{-} \rightarrow Mg_3(PO_4)_2 + 2H^+$$

Similar measurements for Mn^{++} have been made by Chappell et al. (302), and for Ca^{++} by Saris (310).

Of great interest are some findings of DeLuca et al. (295, 296) relating to the effects of parathyroid hormone and vitamin D on mitochondrial ion accumulation. Kidney mitochondria from normal and vitamin D-deficient rats revealed no difference in the rate and extent of Ca^{++} accumulation, provided that this was measured in the presence of a relatively high concentration (*ca.* 2 m*M*) of ATP. However, when the concentration of ATP was lowered by 15-fold, a release of accumulated Ca^{++} from the mitochondria was observed, which in the case of vitamin D-deficient animals could be greatly enhanced by the addition of vitamin D_2 or D_3. The enhanced release of Ca^{++} upon addition of vitamin D occurred also with mitochondria derived from parathyroidectomized animals, and could then be further stimulated by the addition of parathyroid hormone. The hormone alone had no effect on the release of Ca^{++}. In more recent experiments (297) it has been shown that liver mitochondria from parathyroidectomized rats are virtually unable to accumulate Mg^{++} and P_i unless parathyroid hormone is added. The hormone-induced ion uptake was accompanied by a marked increase in the rate of the oligomycin-insensitive respiration. Vitamin D was not required for the hormone effect. From these findings it appears that parathyroid hormone is involved primarily in the active accumulation of Mg^{++} and P_i in the mitochondria, whereas vitamin D acts by promoting the release of Ca^{++} from the mitochondria. Whether the latter effect is a direct one or a secondary consequence of an enhanced breakdown of ATP (which is necessary for protecting the mitochondrial structure against Ca^{++}) remains to be settled. In any case these results seem to open a very promising approach to the mode of action of parathyroid hormone and vitamin D at the subcellular level.

OXIDATIVE PHOSPHORYLATION

COUPLING FACTORS AND HIGH-ENERGY INTERMEDIATES

In recent years the isolation and identification of coupling factors and high-energy intermediates has become a major trend in the study of oxidative phosphorylation. For the first time a symposium has been devoted entirely to this topic.

In 1960, Racker and collaborators (312–314) purified a protein component from extracts of mechanically disrupted beef heart mitochondria which was able to restore phosphorylation to the particulate submitochondrial residue. An increase in P/O ratio from near zero to between 0.5 and 1.0 was obtained with either NADH or succinate as the substrate. No restoration occurred with ascorbate+cytochrome-*c* as substrate. It was hence concluded that the factor specifically restored the phosphorylation at Coupling Site II, although ascorbate+cytochrome-*c* also abolished its restoring effect on the succinate-linked phosphorylation. This factor, called F_1, exhibited an ATPase activity, which followed the activity in restoring phosphorylation during the course of the purification. The ATPase activity was characteristically cold labile, and was insensitive to oligomycin. The factor catalyzed the hydrolysis of ATP, GTP, UTP, and ITP but not CTP. This is the same pattern of specificity later found by Löw et al. (177) for the nucleoside diphosphate requirement in phosphorylation with beef heart electron transport particles. F_1 does not catalyze P_i-ATP or ADP-ATP exchange reaction.

F_1 was obtained from mitochondria after treatment with a Nossal shaker. When the particles so obtained were further treated by sonic oscillation and centrifuged, F_1 no longer could restore phosphorylation unless a second factor, F_2, from the sonic extract was added (312, 314).

A third factor (F_3) was obtained by Racker (315) by sonic treatment of beef heart mitochondria in a pyrophosphate buffer and the exposure of the resulting particles to treatment with trypsin. F_3, which was isolated from the extract obtained after sonic treatment, exhibited P_i-ATP exchange activity, and addition of this factor, together with F_1, was necessary to restore phosphorylation to the trypsin-treated particles.

Sonic treatment of beef heart mitochondria with either ammonia or a preparation of phosphatides led Conover et al. (316, 317) to the recognition of a fourth factor (F_4). This factor, which was purified from the sonic extract and revealed some similarities to the structural protein of Criddle et al. (31), was indispensable for restoring P_i-ATP exchange, oxidative phosphorylation, and the succinate-linked NAD^+ reduction in the ammonia- or phosphatide-treated particles in the presence of F_1. Soluble factors which stimulate the succinate-linked NAD^+ reduction in submitochondrial particles from beef heart have also been described by Hommes (172, 173) and by Sanadi et al. (180–183).

Pullman and associates (318, 319) have recently described the purification of a protein extracted from alkali-treated mitochondria which appears to be a specific inhibitor of mitochondrial ATPase. It inhibits the ATPase activity of submitochondrial particles and of purified F_1 but does not uncouple phosphorylation. An inhibitor-F_1 complex has been isolated which does not hydrolyze ATP but does replace F_1 in recoupling oxidative phosphorylation and restoring succinate-linked NAD^+ reduction in nonphosphorylating submitochondrial particles. The complex does not catalyze a P_i-ATP or ATP-ADP exchange reaction. Whereas the ATPase and coupling activities of F_1 are cold-labile, these activities of the complex are cold-stable. These interesting results may explain the earlier finding of Pullman et al. (312) that addition of F_1 to submitochondrial particles did not increase the ATPase activity of the latter.

Racker (320) found that when particles obtained from beef heart mitochondria by sonic oscillation were treated with trypsin and urea and again subjected to the sonic treatment, a factor F_0, was released which conferred oligomycin sensitivity on the ATPase activity of the purified F_1. An analogous observation was made by Kettman & King (321) who found that an oligomycin-insensitive ATPase extracted from acetone-treated Keilin & Hartree heart muscle preparation resumed oligomycin sensitivity when added to ATPase-free particles. Very recently, Racker (322) also reported that F_0 exhibits NADH- and succinate oxidase activities and that upon combination with F_1 and F_2, a slight but significant phosphorylation (P/O ratio of the order of 0.08) occurred with either substrate. The combined system also exhibited a DNP- and oligomycin-sensitive P_i-ATP exchange activity. This appears to be the first reported instance of a nonparticulate respiratory and phosphorylating system.

The impressive work of Racker and associates is summarized in two symposium papers (323, 324).

In 1958 Linnane (325) reported the pioneering observation that disruption of beef heart mitochondria by sonic treatment in the presence of EDTA resulted in submitochondrial particles ("modified ETP_H") that required a soluble protein fraction for maximal phosphorylation. This factor was purified 8 to 15-fold by Linnane & Titchener (326), who showed that it restored phosphorylation coupled to the oxidation of both succinate and NADH. Evidence was also presented that the restoration of phosphorylation was dependent upon a recombination of the soluble factor and the particle, in the presence of Mg^{++}.

More recently, Webster (327) reported a further purification of this factor by about 250-fold. He also showed that the factor had no effect on the phosphorylation which accompanied the oxidation of NADH and succinate by phenazine methosulfate (PMS) in a system in which the cytochrome oxidase step was blocked by cyanide. In contrast, the phosphorylation accompanying the oxidation of cytochrome-*c* (kept reduced by ascorbate) by cytochrome oxidase in an antimycin A-blocked system was almost entirely dependent upon the presence of the factor. Hence he concluded that the factor was specifically required for the phosphorylation occurring between cytochrome-*c* and oxygen. The factor exhibited an ATP-ADP exchange activity, but was devoid of P_i-ATP exchange and ATPase activities. Later, Webster et al. (147) reported the preparation from beef heart mitochondria of a cytochrome oxidase-containing particle which catalyzes only the phosphorylation step coupled to the aerobic oxidation of reduced cytochrome-*c*. Oxidative phosphorylation by this particle exhibited an absolute requirement for the reduced cytochrome-*c* coupling factor described earlier. During the oxidation of cytochrome-*c* in the presence of the coupling factor and of Mg^{++}, a soluble, nonphosphorylated substance was formed which readily reacted with ADP and P_i to form ATP. The substance was formed only if cytochrome-*c* was oxidized in the presence of the coupling factor; its formation, but not its subsequent interaction with ADP and P_i, was prevented by DNP. Very recently, evidence was presented (328) that when submitochondrial particles (modified ETP_H) were incubated with reduced cytochrome-*c* in the absence of coupling factor, cytochrome-*c* became bound to the particles. It could be released therefrom upon incubation with coupling factor and Mg^{++}, presumably in the form of a "cytochrome-*c* coupling factor" complex. Incubation of this complex with P_i resulted in the formation of free cytochrome-*c* and a phosphorylated coupling factor; the latter, upon further incubation with ADP, yielded ATP and free coupling factor. The following equations were proposed to account for the reaction sequence (RCCF = "reduced cyctochrome-*c* coupling factor"):

$$\text{Particle} + n\ \text{cyt-}c^{++} \rightarrow \text{Particle} \sim \text{cyt-}c + n\ \text{cyt-}c^{+++}$$

$$\text{Particle-cyt-}c + \text{RCCF} \rightarrow \text{RCCF} \sim \text{cyt-}c + \text{particle}$$

$$\text{RCCF-cyt-}c + P_i \rightarrow \text{RCCF} \sim \text{P} + \text{cyt-}c^{+++}$$

$$\text{RCCF} \sim \text{P} + \text{ADP} \rightarrow \text{RCCF} + \text{ATP}$$

Smith & Hansen (329) and, more recently, Green et al. (330) have reported experiments which they interpret as evidence for the isolation of two further coupling factors, which are specific for Coupling Sites I and II of the respiratory chain, respectively. In the experiments of Smith & Hansen (329), phenazine methosulfate, in the absence of cyanide, was used as the electron acceptor for the oxidation of: (*a*) NADH+antimycin A; (*b*) NADH+amobarbital; and (*c*) succinate+antimycin A. It was anticipated that these conditions were suitable for assaying the phosphorylations occurring at: (*a*) Coupling Sites I and II; (*b*) Coupling Site I; and (*c*) Coupling Site II. It was also assumed that reduced phenazine methosulfate in these systems was reoxidized by O_2 nonenzymically with the formation of H_2O_2, and the P/O ratios obtained were corrected accordingly. However, as later shown by Löw et al. (331, 332), the phosphorylation observed under the conditions employed by Smith & Hansen (329) very probably involves neither the first nor the second, but only the third coupling site of the respiratory chain. The reoxidation of reduced phenazine methosulfate and the accompanying phosphorylation were abolished by cyanide, indicating that cytochrome oxidase was involved and no H_2O_2 was formed. Green et al. (330) assayed the coupling factors by measuring the net increase in the phosphate uptake accompanying the aerobic oxidation of NADH, succinate, and reduced cytochrome-*c*, respectively. "Factor I" increased the phosphorylation only with NADH, and "Factor II" with both NADH and succinate, but not with reduced cytochrome-*c*, as the substrate. However, the particles used in these experiments exhibited a rather high phosphorylation efficiency even without added factors. Further work will be required to substantiate these interesting results.

Wadkins & Lehninger (124) have described what would seem to be a site-specific restoration of the terminal respiratory chain phosphorylation by a purified ADP-ATP exchange enzyme. Treatment of rat liver mitochondria with ammonium sulfate in the cold, and subsequent centrifugation, resulted in a solubilization of about 50 percent of the ADP-ATP exchange activity; and simultaneously the P/O ratio of the pellet was lowered by one unit with either 5-hydroxybutyrate or succinate as substrate; with ascorbate+cytochrome-*c*, phosphorylation was lost entirely. Fractionation of the mitochondrial supernatant on diethylaminoethyl cellulose revealed two peaks exhibiting ADP-ATP exchange activity. One of these was active in restoring phosphorylation, with a P/O ratio of approximately one, with ascorbate+cytochrome-*c* and in elevating the P/O from about two to three with β-hydroxybutyrate, and from one to two with succinate as substrate.

Lee et al. (226) have recently compared various types of submitochondrial particles from beef heart with respect to their ability to carry out energy-dependent conversion of $NADH+NADP^+$ into $NADPH+NAD^+$ with either ATP or aerobically generated high-energy intermediates as the source of energy. They found that phosphorylating particles, such as those prepared in the presence of $ATP+Mg^{++}$ or $succinate+Mg^{++}+Mn^{++}$, could carry out the reaction with both kinds of energy source. On the other hand, nonphosphorylating particles such as those prepared in the presence of EDTA,

$EDTA+P_i$, ammonia, or urea could only utilize aerobically generated high-energy intermediates but not ATP as the source of energy. The reaction, supported by aerobically generated high-energy intermediates, was, in accordance with previous findings (222–224), not inhibited by oligomycin and was even stimulated by this agent to varying extents, depending on the type of particles used. From these results it was concluded that the nonphosphorylating electron transport particles studied were capable of respiratory chain-linked generation of nonphosphorylated high-energy intermediates. This appears to be the first demonstration of energy coupling in nonphosphorylating electron transport systems. Thus, what makes these particles nonphosphorylating is not the lack of capacity for energy coupling but only that of transferring energy to form ATP. Since most coupling factors so far described have been used in connection with the types of particles which now have been shown to possess the capcity for energy coupling, these factors probably promote only energy transfer, rather than energy coupling in the true sense.

As early as 1953, Pinchot (333) reconstructed oxidative phosphorylation with a system derived from *Alcaligenes faecalis* by combining a particulate NADH oxidase with a heat-labile soluble component and a heat-stable factor. The heat-stable factor was later shown to consist of an oligoribonucleotide (334, 335). This oligonucleotide, together with Mg^{++}, was needed to combine the heat-labile factor with the oxidase; and it was shown (336) that, during oxidation of NADH, the heat-labile component was again released unless P_i and ADP were present. It was postulated (336) that the released component was present in the form of a high-energy complex, and could not reassociate with the particles unless it was reliberated from the complex by the action of P_i and ADP with the formation of ATP.

Pinchot & Hormanski (337) and Pinchot (146) have recently demonstrated that the high-energy complex of the heat-labile factor is an enzyme~NAD compound. They have isolated the compound and shown that it yields equivalent amounts of NAD^+ and ATP upon incubation with ADP and P_i. Interestingly, DNP did not interfere with the latter reaction, indicating that it did not split the preformed high-energy intermediate. On the other hand, DNP did uncouple the overall phosphorylation, and evidence was presented that its effect consisted of an inhbition of the association of the soluble enzyme with the NADH oxidase. Indications were also reported that the interaction of enzyme~NAD with ADP and P_i to form ATP involves the phosphorylated enzyme as the intermediate. The following reaction sequence was proposed (PON = particulate oxidase-oligonucleotide complex; E = coupling enzyme):

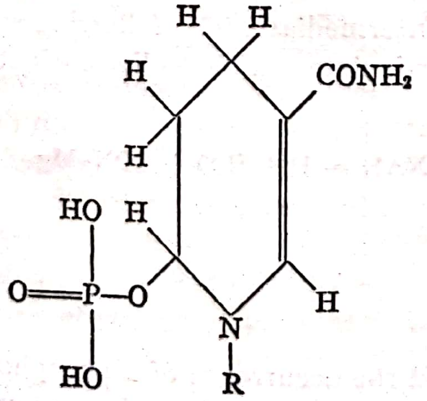
$$\text{PON–Mg}\cdot\text{E} + \text{NADH} + \text{H}^+ + 1/2\ \text{O}_2 \rightarrow \text{NAD} \sim \text{E} + \text{H}_2\text{O} + \text{PON–Mg}$$

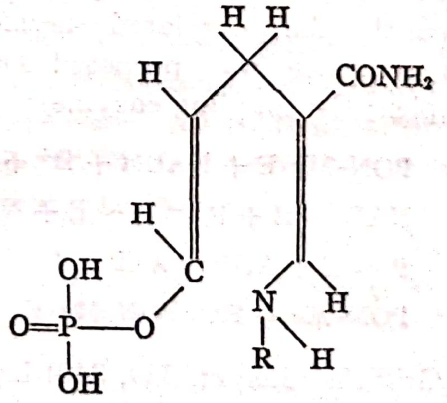
$$\text{NAD} \sim \text{E} + \text{P}_i \rightleftharpoons \text{P} \sim \text{E} + \text{NAD}^+$$

$$\text{P} \sim \text{E} + \text{ADP} \rightleftharpoons \text{ATP} + \text{E}$$

$$\text{PON–Mg} + \text{E} \rightleftharpoons \text{PON–Mg}\cdot\text{E}$$

Griffiths (338; cf. 339, 340) has reported the occurrence of a phosphorylated form of NAD^+, distinct from $NADP^+$. The compound was formed upon aerobic incubation of either mitochondria or submitochondrial particles from beef or sheep heart in the presence of succinate, NAD$^+$, and P_i. It could be isolated, after heat-denaturation of the enzyme, by either paper chromatography or paper electrophoresis, both of which clearly separated it from NAD$^+$ and P_i. The eluted compound was very labile even in neutral solution and decomposed into NAD$^+$ and P_i with a molar ratio of one to one. Closer analysis of the time-course of decomposition revealed a complex sequence of events. Some of the spectral changes resembled those recently observed by Skulachev (341) and interpreted by him as being due to oxido-reduction of the adenine ring of NAD$^+$. The amount of enzymically active NAD$^+$ (as determined by the alcohol dehydrogenase assay) in the decomposed sample was higher initially than the amount of P_i while the relationship was converse upon progressing decomposition. When the phosphorylated NAD$^+$ was incubated with mitochondria or submitochondrial particles, NAD$^+$ and P_i were rapidly formed in a molar ratio of approximately 0.9:1. Spectral analysis of the reaction product revealed the presence of a compound absorbing at 290–320 mμ which did not react in the alcohol dehydrogenase assay. When the incubation was carried out in the presence of antimycin A, NADH (rather than NAD$^+$) and P_i were rapidly formed and the formation of P_i was approximately twice as rapid as that of NADH. When the reaction was carried out in the presence of ATP, hexokinase, and glucose, NADH again was rapidly formed, but glucose-6-phosphate, rather than P_i, was the accompanying reaction product, indicating a transfer of the phosphate group of the phosphorylated NAD$^+$ compound to ADP; the amount of glucose-6-phosphate formed was again twice that of NADH. DNP, amobarbital, antimycin A, and malonate inhibited the formation of the phosphorylated NAD$^+$, but not its subsequent conversion into NADH and P_i or ATP, except that the formation of ATP was partially inhibited by DNP. On the other hand oligomycin did not inhibit the formation of the phosphorylated NAD$^+$, or its subsequent splitting into NADH and P_i, but it did inhibit its conversion into NADH and ATP. The possible structure of the phosphorylated NAD$^+$ was discussed in relation to previously suggested structures for "DPNH-X" (342) and for the acid decomposition product of NADH (343), as well as to the "NAD$^+$ cycle" mechanism proposed by Kaplan in 1951 (344). The following structures were considered [cf. also Barltrop et al. (345)]:



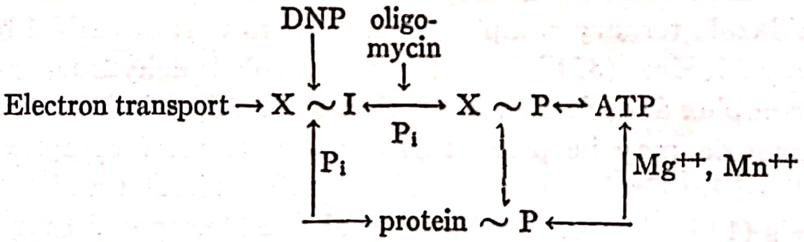


From the evidence presented, Griffiths (338) concluded that the phosphorylated NAD+ has the properties expected of a high-energy intermediate of oxidative phosphorylation: (*a*) it is formed by mitochondria after incubation with succinate; and (*b*) ATP is formed from the compound in an oligomycin-sensitive reaction.

Starting from the observation of Chance & Baltscheffsky (346) that mitochondrially bound pyridine nucleotide exhibits a fluorescence, Estabrook (347) and Avi-Dor et al. (348) independently found that this fluorescence could be considerably more intense than that ordinarily observed with NADH bound to enzyme proteins (cf. 349). Subsequently, Estabrook et al. (257, 350) showed that the extent of this fluorescence enhancement was related to the metabolic state of the mitochondria; it was high in the controlled state, and low in the actively respiring, either phosphorylating or uncoupled, state. These observations are at variance with those previously reported by Chance & Baltscheffsky (346), who have stated that "concentrations of uncoupling agents sufficient to activate respiration and to cause oxidation of RPN [reduced pyridine nucleotide] do not prevent the increase of RPN fluorescence in anaerobiosis." Thus these preliminary studies indicate that the intensified fluorescence may not require a high-energy bond with the carrier (I), and the fluorescent configuration could be either RPN$\sim$I or RPN$\cdot$I; i.e., substance I can be bound to RPN in a low-energy form without inhibiting its oxidation by flavoprotein (cf. also 251). Estabrook et al. (257, 350) discussed the possibility that the greatly enhanced fluorescence in the controlled state may be due to the presence of an enzyme-NADH-imidazole ternary complex, analogous to that described by Theorell & McKinley-McKee (351, 352) for the alcohol dehydrogenase-NADH-imidazole complex and, furthermore, that the NADH-imidazole complex in the mitochondria may be present as a charge-transfer complex similar to that visualized by Grabe (143) to exist between NADH and FAD. In line with Grabe's (143) theory, Estabrook et al. (350) proposed that this charge transfer complex may interact with P_i with the formation of a phosphoimidazole and the liberation of NADH. The phosphoimidazole may correspond to the phosphohistidine of Boyer et al. (353). Although these recent findings of Estabrook et al. (257, 350) undoubtedly provide evidence for a form of bound NADH especially related to the controlled respiratory state of the mitochondria, more work seems to be needed to elucidate its chemical nature and function.

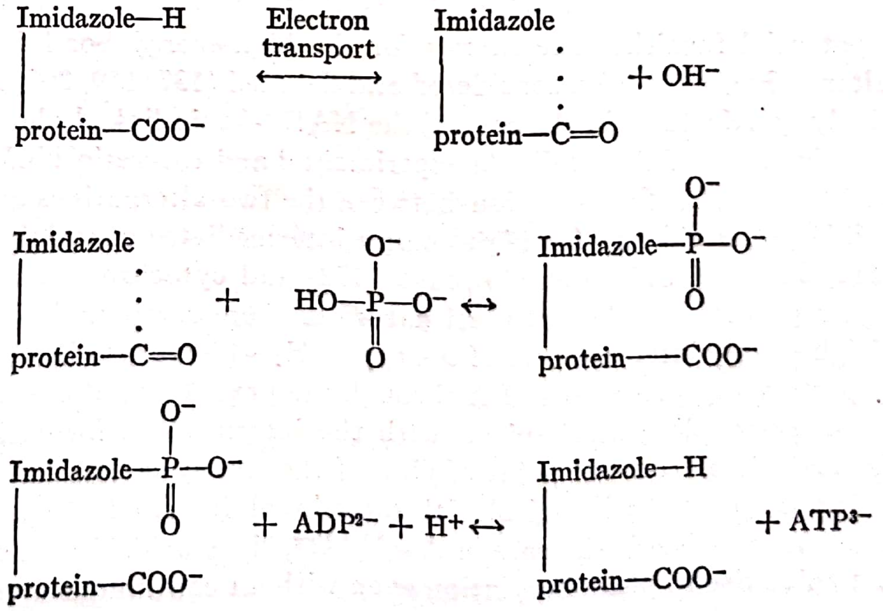
It has been noticed for some time that mitochondria during oxidative phosphorylation rapidly incorporate P_i into phospholipids (357) and phosphoproteins (358), and both classes of compound have been considered as potential intermediates in oxidative phosphorylation. In an interesting recent study with rat liver and rat and hog kidney mitochondria, Garbus et al. (359) found a rapid incorporation of P_i^{32} into an unknown lipid with properties of a phosphorylated analogue of phosphoinositide. The process required no added substrate but was inhibited by anaerobiosis, cyanide, and antimycin A, as well as by DNP and related uncouplers. On the other hand, oligiomycin did not inhibit the incorporation, and the labeling of intramitochondrial ATP also was insensitive to this agent. Regardless of the length of the incubation, the specific activity of the ATP was 15- to 20-fold higher than that of the unknown lipid. Hence it was concluded that this material probably does not function as an intermediate in oxidative phosphorylation.

In 1960, Judah (360) found that P_i^{32} is rapidly incorporated in the phosphoprotein of rat liver mitochondria. Antihistamine drugs inhibited the incorporation of P_i into phosphoprotein but not into ATP. The drugs also inhibited the swelling and contraction of the mitochondria. In subsequent studies, Ahmed & Judah (361, 362) could distinguish two labeled phosphoprotein fractions, one containing phosphoserine, and the other an alkali-labile phosphate. Inhibitors of electron transport and uncouplers reduced the rate of labeling of the phosphoproteins. In the presence of these agents the phosphoproteins failed to reach control levels of radioactivity at any time. The turnover of mitochondrial ATP was also reduced by inhibitors of electron transport and by uncouplers. In contrast to the phosphoproteins, mitochondrial ATP reached the same isotopic equilibrium both in the presence and absence of inhibitors or uncouplers within a few minutes. Oligomycin, however, did not inhibit phosphoprotein turnover at concentrations at which the labeling of ATP was greatly reduced. Ahmed & Judah (361, 362) proposed that mitochondrial phosphoproteins lie on a cyclic pathway between intermediates of the oxidative phosphorylation system and ATP as indicated in the following scheme:



Boyer and associates (353, 363–369) have demonstrated a rapid incorporation in rat or beef liver mitochondria of P_i^{32} into a phosphoprotein. After proteolytic and alkaline degradation of the labeled material, phosphohistidine was isolated and it was shown that the phosphate is attached to N_1 or N_3 of the imidazole ring. The formation of the compound was observed upon incubation of liver mitochondria for a short period of time in the presence of O_2 and trace amounts of highly labeled P_i^{32}. No ADP was added and addition of substrate had no effect. The specific activity of the phosphoprotein was markedly higher initially than that of the intramitochondrial ATP, but equilibrated with the latter upon continued incubation. Addition of unlabeled P_i or ATP diluted the radioactivity of both the phosphoprotein and the intramitochondrial ATP. DNP had only a weak effect on the rate of incorporation of P_i into the phosphoprotein and the endogenous ATP, giving an incomplete inhibition at a concentration as high as 1 m*M*. The appearance of P^{32} in the phosphoprotein was not inhibited by oligomycin and only partially depressed by amobarbital or KCN. It was strongly inhibited by 10^{-5} M Cd^{++} and completely by 1 mM *p*-chloromercuribenzoate.

Labeling of the phosphohistidine-containing protein also occurred when the reaction was started with terminally labeled P^{32}-ATP. The appearance of P^{32} in the bound phosphohistidine was concluded to be more rapid than the appearance of P_i^{32}, in particular when oligomycin was present. Extracts of alkali-treated or frozen and thawed liver mitochondria catalyzed an incorporation of P_i^{32} into protein-bound phosphohistidine, from both P^{32}-ATP and P_i^{32}. The kinetics of the incorporation corresponded to the pattern expected for an exchange between P_i and ATP, with a protein-bound phosphohistidine as an intermediate. An actual P_i-ATP exchange in the extract could in fact be demonstrated. Exposure of the extract to alkaline pH abolished the ability to incorporate P_i into phosphohistidine, while the ability to incorporate phosphate from ATP remained unaltered. The extract was also able to reduce added NAD^+, which was abolished by ADP—hexokinase-glucose in the presence of P_i. Hydroxylamine blocked both the NAD^+ reduction and the incorporation of P_i into bound phosphohistidine, but not the formation of the latter from ATP. These findings suggested that the extract contained an active form of histidine which was an intermediate energy carrier between the respiratory chain and the generation of phosphohistidine and ATP. It was proposed that protein-bound histidine may combine with various electron carriers of the respiratory chain during electron transport, with the subsequent formation of a covalent bond between a carboxyl C-atom of the protein and one of the N-atoms of the imidazole ring. This "activated" histidine would then react with P_i to form N-phosphohistidine. The phosphate group of the latter would finally be transferred to ADP:



$$\begin{array}{l}\text{Imidazole—H}\\ |\\ \text{protein—COO}^-\end{array} \xleftrightarrow{\text{Electron transport}} \begin{array}{l}\text{Imidazole}\\ | \quad \vdots\\ \text{protein—C=O}\end{array} + \text{OH}^-$$

$$\begin{array}{l}\text{Imidazole}\\ | \quad \vdots\\ \text{protein—C=O}\end{array} + \text{HO—}\overset{\overset{\text{O}^-}{|}}{\underset{\underset{\text{O}}{\|}}{\text{P}}}\text{—O}^- \leftrightarrow \begin{array}{l}\text{Imidazole—}\overset{\overset{\text{O}^-}{|}}{\underset{\underset{\text{O}}{\|}}{\text{P}}}\text{—O}^-\\ |\\ \text{protein—COO}^-\end{array}$$

$$\begin{array}{l}\text{Imidazole—}\overset{\overset{\text{O}^-}{|}}{\underset{\underset{\text{O}}{\|}}{\text{P}}}\text{—O}^-\\ |\\ \text{protein—COO}^-\end{array} + \text{ADP}^{2-} + \text{H}^+ \leftrightarrow \begin{array}{l}\text{Imidazole—H}\\ |\\ \text{protein—COO}^-\end{array} + \text{ATP}^{3-}$$

REACTION MECHANISMS

It is now firmly established that there occur at least three sites of phosphorylation in the respiratory chain, one between NADH and cytochrome-*b*, a second between cytochrome-*b* and cytochrome-*c*, and a third on the path of electrons from cytochrome-*c* to oxygen. Each of these spans of the chain includes several electron transfer steps, and it is not known with certainty within exactly which step the energy coupling takes place for any of the three sites of phosphorylation. The $NAD^+ \rightarrow$flavin step is usually considered as the site of the first energy coupling, but the fact that the reduction of both the NADH dehydrogenase flavin (197) and coenzyme Q (231) by flavin-linked substrates is "energy dependent" suggests a more complex pattern. The possibility that this phosphorylation may extend over several electron transfer steps in sequence has been considered (143). As for the cytochrome-*b*→cytochrome-*c* span, the possible involvement, in energy coupling, of the nonheme iron in this span may deserve future attention, especially since it now appears that this catalyst may be the site of action of antimycin A (57, 60). In the terminal span, cytochrome-$c \rightarrow O_3$, the energy coupling has been considered to occur either between cytochrome-*c* and cytochrome oxidase (9), or between the latter and O_2 (252), or even in both steps (66, 67, 68); in the last case, the total number of energy coupling sites would be four.

The nature of the primary high-energy intermediates is not yet clarified. The demonstration that an energy transfer between the individual coupling sites of the respiratory chain can take place in the absence of phosphate (211) has substantiated the concept that energy coupling proceeds by way of nonphosphorylated high-energy intermediates (370); these are denoted as $C{\sim}I$ (carrier$\sim$I). On the other hand, it is not known whether these intermediates contain the electron carrier in the oxidized or the reduced form. Logically, C in $C{\sim}I$ ought to be identical with either A or BH_2 in the redox reaction,

$$AH_2 + B \rightleftharpoons A + BH_2$$

whose potential furnishes the energy for the high-energy bond formation. Both alternatives have been considered and debated (137, 169, 203, 211, 252) extensively, particularly in the case of the NAD^+-flavin-linked phosphorylation to which most of the available experimental and theoretical information relates. However, no final decision between the two alternatives appears to be possible yet. Evidence for high-energy intermediates containing NAD^+ (279, 280, 338–340, 371), cytochrome-*b* (372) and cytochrome-*c* (147, 328) has been reported, but the chemical nature of these compounds in still unclear. High-energy intermediates of the type $AH_2{\sim}I$ have also been proposed (9), by postulating a recycling of A through the preceding redox step as part of the energy-coupling mechanism, with the intermediate formation of an energized A. Thus, from the point of view of the energy–conserving step,this mechanism is essentially identical with those involving $A{\sim}I$. A special problem in this mechanism is the case of $A = NAD^+$, since oxidation of NADH is known to give rise to phosphorylation even without continuous regeneration from NAD^+, i.e., without the possibility for recycling, and since NAD^+ can be reduced by succinate with the expenditure of one high-energy bond per molecule of NAD^+ reduced (74).

The reaction pathways leading to the formation of ATP are three in number:

$$C \sim I + X \rightleftharpoons C + I \sim X \tag{1.}$$

$$I \sim X + P_i \rightleftharpoons I + X \sim P \tag{2.}$$

$$X \sim P + ADP \rightleftharpoons X + ATP \tag{3.}$$

The intermediate, $I \sim X$, was originally postulated by Chance & Williams (9), who considered it to be the site of action of DNP. While this view is no longer maintained (139), there is now another reason for postulating an intermediate of the type $I \sim X$, namely, the demonstration that DNP and arsenate act at two different levels of the energy transfer system, as revealed by studies with oligomycin. As mentioned earlier oligomycin probably acts by inhibiting Reaction 2, which accounts for its inhibition of the arsenate-uncoupled, but not the DNP-uncoupled, respiration.

It is generally assumed that P_i rather than ADP is the first reactant with the nonphosphorylated high-energy intermediate. This concept rests on the finding (373) that the terminal bridge oxygen of ATP originates from ADP. It has been pointed out (211, 177) that a bound, compartmentalized ADP (or another nucleoside diphosphate) might react prior to P_i, i.e., that X in the present scheme might be identical with a nucleoside diphosphate. This possibility cannot be eliminated with the evidence available and will require further investigation.

Reactions 2 and 3 may be elaborated further to illustrate the fate of the oxygen atoms of P_i (P_i written as $HO-P_i$, and ADP as $HO-ADP$):

$$I \sim X + HO-P_i \rightleftharpoons IOH + X \sim P \tag{4.}$$

$$X \sim P + HO-ADP \rightleftharpoons XH + P \sim O-ADP \tag{5.}$$

Reactions 4 and 5 account for the observation that the exchange of oxygen atoms between P_i and H_2O in mitochondria is more rapid than the net rate of phosphorylation (374) and the exchange of phosphate between P_i and ATP (63). The P_i-ATP exchange involves Reactions 4+5, whereas the P_i-H_3O exchange involves Reaction 4 followed by either a direct dissociation of IOH:

$$IOH + HOH \rightleftharpoons IOH + HOH$$

or a recycling of IOH via $C \sim I$ (C written as CH):

$$IOH + CH \rightleftharpoons C \sim I + HOH$$

Reactions 4 and 5 also account for the recent findings of Itada & Cohn (156) that mitochondria catalyze a rapid exchange of oxygen atoms between arsenate and water, and that the oxygen atoms of arsenate do not appear in

P_i in the course of the arsenolytic ATPase reaction of mitochondria. The arsenolysis of I$\sim$X may be visualized to proceed by the ractions (arsenate $= HO-As_i$):

$$I \sim X + HO-As_i \rightleftharpoons IOH + X \sim As$$
$$X \sim As + HOH \rightarrow XH + HO-As_i$$

with a subsequent exchange of oxygen between IOH and water either by direct dissociation or by recycling via C$\sim$I as indicated above.

This Scheme, in its present form, does not account for the sensitivity of the ADP-ATP exchange reaction to DNP and oligomycin, and for the inhibitory effect of Mg^{++} on the ATP-supported reversal of electron transport. Both of these features are observed with mitochondria only, and not with submitochondrial particles. Further qualitative changes in the properties of the energy transfer system upon disruption of the mitochondria are the appearance of a Mg^{++}-activated ATPase reaction, the loss of respiratory control, the loss of the strict specificity of the phosphorylating system for adenine nucleotide, and the loss of atractyloside sensitivity. Partial explanations for this modification of the energy transfer system have been suggested by Vignais et al. (122) to account for the loss of the atractyloside sensitivity, and by Wadkins & Lehninger (125) to explain the altered properties of the ADP-ATP exchange reaction. A more general explanation may be based on the assumption that in the intact mitochondria ADP may be firmly associated with the transphosphorylating enzyme (i.e., X) and that this binding site is lost upon disruption of the mitochondria. A competitive relationship between ADP and atractyloside has been demonstrated by Bruni et al. (121).

To account for the direct, nonphosphorylative, transfer of high-energy bonds between the individual coupling sites of the respiratory chain, two types of mechanism, in principle, are conceivable. One is that this transfer proceeds via a common I$\sim$X, utilizing the pathways involved in oxidative phosphorylation, or, otherwise, a special pathway must be postulated, such as (*a*) a direct transfer of I between different C's, e.g.:

$$C_1 + C_2 \sim I \rightleftharpoons C_1 \sim I + C_2$$

or (*b*) a transfer of X between different I's (assuming that there is one special I for each C), e.g.:

$$I_1 + I_2 \sim X \rightleftharpoons I_1 \sim X + I_2$$

Utilization of the pathways involved in oxidative phosphorylation is undoubtedly the simplest assumption. Perhaps the first type of special pathway may appear even simpler, since it does not involve a second component, X. On the other hand, it involves an interaction of two nonadjacent electron carriers. Considering that these carriers may be C_1 and C_3, this does not appear likely. The second type of special pathway involves the existence of three different compounds-I. This has been proposed (375, 376) on the basis

M. Morini

of conclusions drawn from pH effects on the DNP-induced ATPase. Although these conclusions were later revised (137), the concept of three different compounds-I has been maintained, primarily to avoid the postulation of a common reactant for three electron carriers with widely different chemical properties. However, Boyer (367) has pointed out that an imidazole derivative, for example, might well react with structures as different as pyridine nucleotide, flavin, and heme. Existence of three different I's is implicit in the hypotheses of Hülsmann (138) and Chance & Hollunger (96, 131) concerning the action of electron and energy transfer inhibitors. It is visualized that compounds such as amobarbital, guanidine, or antimycin A, which are site-specific, bind I in $C \sim I$ and, consequently, that I ought to be site-specific. On the other hand, there seems to be no compelling evidence that these compounds bind the I, and not the C, moiety of $C \sim I$. It is conceivable that only nonsite-specific uncoupling agents, such as DNP, react with I. But even in the case of these uncouplers the possibility exists, as was pointed out earlier in this chapter, that they compete—rather than react—with I, preventing its coupling to C, that is, that they react with C.

The energy-dependent interaction of NADH and NADP$^+$ to yield NAD$^+$ and NADPH has been shown to proceed at the expense of either ATP or high-energy intermediates generated by the aerobic oxidation of NADH or succinate (222–224). Oligomycin inhibits the ATP-supported reaction, but not that supported by aerobically generated high-energy intermediates. Conversely, DNP inhibits the aerobically supported reaction strongly, but the ATP-supported one only weakly. Guanidines do not inhibit the reaction in either case (260). From these lines of evidence, $I \sim X$ appears to be the most likely site of the energy-transfer system which provides the energy for this process. $NADH \sim I$ or $NADP^+ \sim I$, of the types described by Chance and associates (9, 257, 346, 350) and by Purvis (280), may further be involved as intermediates in the energy-dependent pyridine nucleotide interaction. Furthermore, $NADH \sim P$, as postulated in this Scheme, might be the compound which Griffiths (338) has described. A possible rearrangement of the pyridine nuleotide molecules as part of the energy-dependent pyridine nucleotide interaction has been considered by Ernster & Lee (263).

The active uptake of Ca^{++}, Mg^{++}, and Mn^{++} into the mitochondria has been demonstrated to require high-energy intermediates generated by the respiratory chain (292–305). The uptake of these cations also requires, or is greatly enhanced by, the simultaneous uptake of P$_i$, and they are deposited in the mitochondria as the corresponding phosphates. The three energy-coupling sites of the respiratory chain appear to be equally efficient in supporting active ion uptake (304, 305), and the process is insensitive to oligomycin. These features suggest that, as in the case of the energy-dependent pyridine nucleotide interaction, $I \sim X$ is the high-energy intermediate which provides the energy for the active ion uptake. The fact that P$_i$ is the common counter-anion for the accumulation of the various cations [except for a small degree of Mn^{++} uptake which has been observed in the absence of P$_i$

(302)] makes it logical to assume that $I{\sim}X$ first reacts with P_i, with the formation of a phosphorylated intermediate, and the cation uptake follows in a secondary manner. This is also strongly suggested by the finding (304, 305) that P_i, and not the cation, bears a one to one relationship to the number of high-energy intermediates expended. The phosphorylated intermediate cannot be $X{\sim}P$, because of the oligomycin-insensitivity of the ion uptake; and it is therefore reasonable to visualize that it is an alternative product of the reaction between $I{\sim}X$ and P_i, namely, $I{\sim}P$.

The reports of Ahmed & Judah (361, 362) and of Boyer et al. (353, 363–369) on an oligomycin-insensitive incorporation of P_i^{32} into phosphoproteins, notably bound phosphoserine and phosphohistidine, raises the question as to the relationship of the hypothetical $I{\sim}P$ to either or both of these compounds; a reversible transition between the two types of phosphoprotein as formed in mitochondria has been noticed by Wadkins (377). A possible identity of these phosphoproteins with $I{\sim}P$ is suggested by the findings of Judah (360) that antihistamines which block ion transport inhibit the formation of phosphoprotein but not the formation of ATP. The postulate of Boyer et al. (353, 365–369) that bound phosphohistidine is an intermediate in energy transfer between the respiratory chain and ATP, i.e., is identical with $X{\sim}P$ according to the present terminology, is difficult to reconcile with their finding that the formation of this compound from P_i is insensitive to oligomycin. In fact, the location of $I{\sim}P$ in the present Scheme is not inconsistent with the principal features reported by Boyer et al. (cf. 368) for bound phosphohistidine, namely, that: (*a*) is it labeled more rapidly from P_i^{32} than is intramitochondrial ATP; (*b*) it is labeled more rapidly from ATP^{32} than is intramitochondrial P_i; (*c*) labeling from P_i is inhibited by dinitrophenol; (*d*) labeling from ATP is not inhibited by dinitrophenol; (*e*) it is in dynamic interchange with both P_i and ATP.

A further point of interest is the relationship of phosphoprotein to intramitochondrial ATP. Data of Peter & Boyer (365) indicate two types of interaction between bound phosphohistidine and ATP: an oligomycin-sensitive reaction, involving extramitochondrial ATP, and one insensitive to oligomycin, which takes place between phosphoprotein and intramitochondrial ATP. Peter & Boyer (365) have further found that intramitochondrial ATP differs from added ATP in that its labeling from P_i^{32} is insensitive to oligomycin as well as to uncoupling agents. Oligomycin-insensitive labeling of intramitochondrial ATP from P_i^{32} has also been noticed by Garbus et al. (359) in their recent study of mitochondrial phospholipid synthesis. Lack of effect of DNP on the incorporation of P_i^{32} into intramitochondrial ATP had earlier been reported by Azzone & Ernster (378), who concluded that this ATP originates from the α-ketoglutarate-linked substrate-level phosphorylation. This phosphorylation is insensitive to oligomycin (379). Charles et al. (380) have essentially confirmed the results of Azzone & Ernster (378) but pointed out that, besides ATP, another intramitochondrial compound incorporates P_i^{32} in a DNP-insensitive manner. Ernster (211) has also

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noticed that the level of intramitochondrial ATP in the presence of DNP can be influenced by added succinate in a malonate-insensitive fashion, indicative of involvement of the reaction

$$\text{ATP} + \text{succinate} + \text{CoA} \rightleftharpoons \text{ADP} + \text{P}_i + \text{succinyl} \sim \text{CoA}$$

This reaction may be responsible for the oligomycin- and uncoupler-insensitive intramitochondrial P_i-ATP exchange reaction observed by Peter & Boyer (365). Taken together, the available evidence points to the possibility that, in addition to the reaction pathways considered in the previous paragraphs, there might exist a direct interaction between phosphoprotein and a special pool of intramitochondrial ATP originating from substrate-level phosphorylation.

Energy-requiring mitochondrial functions utilizing directly the intermediates of the respiratory chain-linked energy transfer system may not be restricted to pyridine nucleotide interaction and ion accumulation. Recent reports that the electron transport-dependent swelling of mitochondria is oligomycin insensitive (381) and, conversely, that the ATP-induced mitochondrial contraction is oligomycin sensitive (382) suggest a close relationship of the active uptake and extrusion of water to respiratory chain-linked high-energy intermediates. An intriguing phenomenon, also, is the widely studied incorporation of amino acids into mitochondrial protein which is respiration dependent and DNP sensitive, but oligomycin insensitive (383–387). Particular caution in this case is warranted, however, in view of the very small amounts of amino acid incorporated which make it important to eliminate bound ATP as the source of energy [analogous to the case of the phospholipid synthesis observed by Garbus et al. (359)]. Finally, the realization that respiratory energy can be utilized for purposes other than ATP synthesis raises the question as to whether ATP formation, i.e., P/O ratio, is a true measure of the efficiency of the respiratory energy-coupling system. The long-standing problem of whether the passage of one or two electrons through the respiratory chain is required for the generation of three high-energy bonds has recently been restressed by Boyer (367) and appears to deserve serious consideration.

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ERRATA

Page	Line(s)	Stands:	Should stand:
729	8-9	A survey of the chemistry and function of coenzyme Q has also been published recently (5).	Surveys of the chemistry and function of coenzyme Q (5) and of flavoproteins (6) have also been published recently.
751	19	observation (211)	observation (74)
	24	concluded (211)	concluded (74)
755	22	Klingenberg & Schollmeyer et al.	Klingenberg & Schollmeyer
762	36	Chappel	Chappell
764	36	$2HPO_4^-$	$2HPO_4^=$
765	22	For the first time a symposium	For the first time a symposium (311)
	32	F_i	F_1
768	33	5-hydroxybutyrate	β-hydroxybutyrate
769	31	P_1	P_i
774	16	O_3	O_2
	24	these are denoted as C~I	these are denoted below as C~I
775	4-5	The reaction pathways leading to the formation of ATP are three in number:	The reaction pathways leading to the formation of ATP probably involve three steps:
777	3	compounds-I	compounds I
778	30-31	Furthermore, NADH~P, as postulated in this Scheme, may be the compound which Griffiths (338) has described.	Furthermore, the NADH~P described by Griffiths (338) may be a compound related to this reaction.